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(54) Title: FUNCTIONALIZED POLYMERS FOR SITE-SPECIFIC ATTACHMENT

(57) Abstract

Provided are organic polymers, e.g. poly(ethylene glycol), functionalized with an amino-oxy oxime-forming group and methods for their preparation and use in site-specific, chemoselective ligation to an aldehyde(or ketone)-functionalized target macromolecule or surface under mild ligation conditions. Multi-polymer-containing amino-oxy-functionalized or aldehyde(or ketone)-functionalized polymer constructs are also provided that allow site-specific, chemoselective ligation under mild conditions of the construct (and thus all of its polymers) to a single site on a target macromolecule via an oxime bond. Families of functionalized polymer constructs are also provided wherein each construct differs in topology but not in molecular weight (average) from the others in the same family. Methods for their use include the systematic modification of a target macromolecule to rapidly create a family of target molecules, preferably biologically important proteins, differing in topology but not molecular weight, from which family can be identified macromolecules having desired biological or physical properties, such as enhanced pharmacokinetic behavior.

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FUNCTIONALIZED POLYMERS FOR SITE-SPECIFIC ATTACHMENT

INTRODUCTION

Technical Field

The present invention relates to the preparation of functionalized polymers. More particularly, the present invention relates to reagents and methods for the site-specific chemical modification of target molecules, e.g. biomacromolecules, particularly biologically important polypeptides, and other polydisperse macromolecules such as plastics (e.g., polyethylene or nylon), by means of covalent attachment of functionalized polymers, particularly polyalkylene oxide polymers.

Background

Preparations of polyethylene glycol-modified polypeptides have reduced immunogenicity and antigenicity and also have a longer lifetime in the bloodstream as compared to the parent polypeptides (Abuchowski and Davis (1981) "Enzymes as Drugs", Holcenberg and Roberts, eds., pp367-383, John Wiley & Sons, N.Y.). These beneficial properties of the modified polypeptides make them very useful in a variety of therapeutic applications, such as enzyme therapy. To effect attachment of polyethylene glycol ("PEG") to a protein, the hydroxyl end-groups of the polymer must first be converted into reactive functional groups. This process is frequently referred to as "activation" and the product is called "activated PEG." Methoxypolyethylene glycol ("mPEG") derivatives, capped on one end with a protecting group and bearing on the other end an electrophilic that is reactive towards amines on a protein molecule have been used in most cases. One of the most common form of activated PEG used for preparation of therapeutic enzymes is methoxy-poly (ethylene glycol) succinoyl-N-hydroxysuccinimide ester (Abuchowski et al. (1984) Cancer Biochem. Biophys. 7:175-186). However, one major drawback is that ester linkage between the polymer and succinic acid residue has

limited stability in aqueous media. The use of PEG-phenylcarbonate derivatives for preparation of urethane-linked PEG-proteins was reported (Veronese, et al., 1985). However, the hydrophobic phenol residues (p-nitrophenol or 2,4,5-trichlorophenol) are toxic and have affinity for proteins.

The attachment of reporter' groups, ligands, etc. to proteins usually proceeds in a random fashion through the primary amino group on the side chains of the lysine residues. Several PEG-derivatives have been synthesized that couple with lysine residues. The hydroxyl end groups of PEG are typically converted to reactive functional groups capable of covalent attaching PEG to a protein of interest. These PEG-derivatives include: 2 (alkoxypolyethyleneglycoxy)-4, 6-dichlorotriazine (Abuchowski et al. (1977) J. Biol. Chem. 252:3578-3581); 2-(alkoxypolyethyleneglycoxy)-N-succinimidyl succinate (Abuchowski et al. (1984) Cancer Biochem. Biophys. 7:175-186); 2-(alkoxypolyethyleneglycoxy)-2,2,2-trifluoroethane (Delgado et al. (1990) Biotech. Applied Biochem. 12:119-128); 2-(alkoxypolyethylenealdehyde (Andres et al. (1990) Biotech. Tec. 4:49-54); 2-alkoxypolyethyleneglycoxymethylepoxide (Andrews et al. (1990) Biotech. Tec. 4:49-54). EP539167 (1993) reports PEG modified with a terminal imidate group for reaction with amino groups of proteins.

A major drawback with all the above methods of protein PEGylation is that the polymer conjugations occur randomly and non-site specifically, typically at amino groups, e.g. lysine side chains and the N-terminal amino group, such that the multiple polymer groups can potentially interfere with biological activity, e.g., substrate binding or receptor binding, and such that preparations of conjugates are typically heterogeneous, particularly in view of the fact that the reactions are random and not usually complete due to the preferential activity or accessibility of the various target residues. Moreover, charge alteration of the target molecule is typically caused by the coupling reaction, particularly in polypeptides upon modification of free amino groups. Changes in charge, either upon the introduction of charge to an uncharged residue on an amino acid residue or upon removal or masking of a charge present on an amino acid residue, may adversely affect the biological activity of a protein by several mechanisms including the disruption of the tertiary structure and the destruction of active sites. Such changes in charge are

typically cumulative as derivatization is carried to completion. Typically, increasing the number of polymers per molecule of polypeptide using the methods above very often decreases sterically the accessibility of the active site and thus diminishes biological activities possessed by the unmodified polypeptide. Polyethylene glycol modified TNF-α is a typical example where extensive modification resulted in the complete loss of bioactivity (Tsutsumi et al. (1994) Jpn. J. Canc. Res. 85, 9-12).

In light of the many potential applications of polymer conjugated molecules, and particularly in view of the art's focus on PEG-polypeptides, there is a need in the art to increase the repertoire of functionalized polymers for use in modifying target macromolecules or materials, such as surfaces. Accordingly, there is a need in the art for functionalized polymers in addition to functionalized PEG, as well as methods for allowing one to overcome the disadvantages inherent in and observed for the current methods of polymer attachment, both for medical and non-medical uses. For example, there is a need in the art to provide functionalized polymers that are capable of coupling to residues of target molecules, e.g. amino acid residues of polypeptides, without drastically altering the charge present on the residue or without introducing groups at locations likely to interfere with the binding properties of the target molecule, and that are attached through linkages that are stable under a variety of conditions, particularly physiologically relevant conditions. Thus, a need exists for reagents and methods for constructing homogeneous preparations, of easily synthesized macromolecules of defined structure having stable ligation linkages, wherein these reagents and methods provide ease, rapidity and mildness of synthesis; essentially quantitative yields; versatility in design; and applicability to construction using a diversity of biochemical classes of compounds. The reagents and methods provided in the present invention provide meet these needs and others as well.

SUMMARY OF THE INVENTION

The present invention provides methods and compounds for site-specifically, chemoselectively modifying inder mild conditions a target macromolecule, such as proteins, peptides, other organic compounds such as plastics, or surfaces containing macromolecules, with an amino-oxy or aldehyde (or

ketone) derivative of an organic polymer, preferably a water organic soluble polymer, more preferably PEG or dextran, via an oxime linkage at a unique site on the target macromolecule. Novel amino-oxy derivatives of PEG and other water-soluble polymers, referred to as "functionalized" polymers, are provided that are capable of oxime-linkage formation. Through the use of bi- or multi-polymer-containing functionalized polymers of the invention, one or more water-soluble polymers may be coupled site-specifically and chemoselectively to a single, pre-chosen site on an individual target macromolecule under mild conditions.

Another aspect of the subject invention is to provide a process for preparing proteins modified by the site-specific, covalent attachment of the functionalized water-soluble polymer derivatives. The present invention provides a functionalized polymer wherein the number of polymer repeating units is an integer between 5 and 2000.

The present invention also provides a process for preparing the functionalized polymer that contains an oxime-forming group which includes the step of reacting a polymer compound, wherein the number of polymer repeating units is an integer between 5 and 2000, with a compound containing an oxime-forming group, which oxime-forming group is optionally in a deprotectable form, wherein the oxime-forming group is covalently attached to the polymer.

The present invention further provides a functionalized modified target molecule, preferably a polypeptide, having an oxime-forming reactive group complementary in reactivity to a functionalized polymer of the invention, such that site-specific reactivity and oxime-linkage formation will occur.

The present invention further provides a modified target macromolecule, i.e. a polymer conjugate, preferably comprising a polypeptide, having bound site-specifically thereto at least one organic polymer wherein the polymer or polymers are covalently bound through a single oxime linkage to a unique site on the target macromolecule.

The invention also provides a process for preparing a polymer conjugate described above under mild conditions which includes the steps of functionalizing the target macromolecule to provide a first oxime-forming functional group complementary in activity with a second functional oxime-forming group

present on a functionalized polymer of the invention, reacting the functionalized target macromolecule with the functionalized polymer such that an oxime bond is formed between the first and second functional groups.

Multi-polymer-containing amino-oxy-functionalized or aldehyde(or ketone)-functionalized polymer constructs are also provided that allow site-specific, chemoselective ligation under mild conditions of the construct (and thus all of its polymers) to a single site on a target macromolecule via an oxime bond. Families of functionalized polymer constructs are also provided wherein each construct differs in topology but not in molecular weight (average) from the others in the same family. Methods for their use include the systematic modification of a target macromolecule to rapidly create a family of target molecules, preferably biologically important proteins, differing in topology but not molecular weight, from which family can be identified macromolecules having desired biological or physical properties, such as enhanced pharmacokinetic behavior. The present invention thus provides methods for systematically modifying the Stokes radius of a target macromolecule, which method includes the steps of conjugating a functionalized target macromolecule with a series of functionalized polymers of the invention, preferably the multi-polymer-containing functionalized polymers, in separate reactions, and then determining the effect of conjugation on the Stokes radius. In a preferred embodiment of this aspect of the invention the pharmacokinetic behavior of a target macromolecule is systematically modified.

Kits are provided with appropriate reagents for carrying out the methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an SDS-PAGE gel under non-reducing conditions showing the migration of conjugates of oxidized Il-8 with different functionalized PEG polymers. In all cases a single derivative was obtained in the conjugate reaction. Lanes 1, 3, 5, 7: Result of 20 hour incubation of oxidized IL-8 with functionalized AoA-PEG_{5kD}, AoA-PEG_{10kD}, AoA-PEG_{20kD}, and AoA-N-$(PEG_{5kD})_2$, respectively. AoA-$N(PEG_{5kD})_2$ is $(PEG_{5kD}\text{-}CONH(CH_2)_2)_2N\text{-}(CH_2)_2\text{-}NH\text{-}AoA$.

Lanes 2, 4, 6, 8: purified conjugates PEG_{5kD}-Il-8, PEG_{10kD}-Il-8, PEG_{20kD}-Il-8 and $(PEG_{5kD})_2$-Il-8. Lane 9, oxidized Il-8,; Lane 10, protein molecular weight markers: from top to bottom: Phosphorylase B (97.4 kD), bovine serum albumin (66.2kD), ovalbumin (45kD), carbonic anhydrase (31kD), soybean trypsin inhibitor (21.5 kD), lysozyme (14.4kD). "kD" indicates kilo Daltons.

Figure 2 depicts an SDS-PAGE gel showing migration of Il-8 conjugates obtained with the different functionalized polymers. Lane 1: Il-8. Lanes 2, 3, 4: PEG_{5kD}-Il-8, PEG_{10kD}-Il-8, PEG_{20kD}-Il-8. Lane 5: Il-8-PEG_{20kD}-Il-8 (dimer; "dumbbell"). Lane 6, 7: $(PEG_{10kD})_2$Lys-Il-8, $(Lys\text{-}PEG_{5kD})_5$-Il-8. Lane 8: $Dextran_{9kD}$-Il-8. Lane 9: Protein molecular weight markers are the same as in Figure 1. PEG_{5kD}-PEG_{10kD}-, PEG_{20kD}-Il-8, Il-8-PEG_{20kD}-Il-8 and $(PEG_{10kD})_2$Lys-Il-8 migrated as a single band, while $(Lys\text{-}PEG_{5kD})_5$-Il-8 and $Dextran_{9kD}$-Il-8 gave a trail on the gel.

Figure 3 depicts an SDS-PAGE gel showing migration of conjugates of functionalized PEG_{5kD} and PEG_{20kD} with *des-Met*-G-CSF. Lane 1: *des*-Met-G-CSF. Lane 2: result of 24h incubation of oxidized *des*-Met-G-CSF with AoA-PEG_{5kD}. Lane 3: Purified PEG_{5kD}-*des*-Met-G-CSF. Lane 4: Coupling of AoA-PEG_{20kD} to oxidized *des*-Met-G-CSF after 24h incubation. Lane 5: Purified PEG_{20kD}-*des*-Met-G-CSF. Lane 6: Protein molecular weight markers are as in Figure 1.

Figure 4. Conjugation of functionalized AoA-PEG_{5kD}, AoA-PEG_{10kD} and AoA-PEG_{20kD} to Interleukin-1 Receptor Antagonist Protein ("IL-1ra"). Lane 1: Il-1ra. Lane 2: Coupling of AoA-PEG_{10kD} to transaminated Il-1ra after 48h incubation at room temperature. Lanes 3, 4, 5: Purified conjugates PEG_{10kD}-Il-1ra, PEG_{5kD}-Il-1ra, and PEG_{20kD}-Il-1ra. Lane 6: Protein markers are as in Figure 1.

Figures 5A, 5B and 5C depict dose-response curves showing inhibition of IL-1β induced Prostaglandin E2 production by dermal fibroblasts in the presence of decreasing concentrations of Il-1ra derivatives. Figure 5A is that for $PEG_{(5kD)}$-Il-1ra, Figure 5B is that for $PEG_{(10kD)}$-Il-1ra, and Figure 5C is that for $PEG_{(20kD)}$-Il-1ra. In each case the conjugate was prepared as described in Example 8 using the transamination to provide an activated protein. Each point was derived from a mean of three prostaglandin determinations, from which was subtracted the mean of three prostaglandin determinations obtained when buffer only was used in

place of the Il-1ra (also designated herein as "Antril" (from Synergen, Inc.)) and derivatives. The dispersion of the means is not unusual in this assay. For clarity, the dose-response curves for the three Antril derivatives were plotted separately (dotted lines and error bars), each time with the same standard curve obtained with Antril (solid line and error bars).

Figures 6A and 6B depict pharmacokinetics of PEG_{10kD}-Il-1ra (Figure 6A) and PEG_{20kD}-Il-1ra (Figure 6B) in rats, compared with that of unmodified Il-1ra. For clarity, the results with the two derivatives are plotted separately (dotted lines), each time with the same set of results obtained for unmodified Antril (solid line). Blood samples were collected at 3, 10, and 30 minutes and at 1, 3, 7, 12 and 24 hours. Each point represents a single determination with a single animal. In case of unmodified Antril, a slow rise in blood levels after an initial decrease could be shown between 30 and 180 min. Such an unexpected behavior was not observed with other investigated proteins used under the same experimental conditions. Animals that showed clear evidence that some or most of the injection had missed the tail vein and had been deposited subcutaneously (excessive radioactivity remaining at the injection site followed by a slow rise in blood levels after an initial decrease rather than the usual biphasic, exponential-like decrease) were discarded.

Figure 7 depicts pharmacokinetics of Il-8, PEG_{20kD}-Il-8, Il-8-PEG_{20kD}-Il-8 and $(PEG_{20kD})_2$Lys-Il-8. Blood samples were collected at 3, 7, 15 and 30 minutes, and at 1, 3 and 7 hours. Each point represents the mean value or four animals. The dumbbell and the multiarm constructs appear as the most effective conjugates for improving the lifetime of Il-8 in the bloodstream.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The subject invention provides novel modifying reagents that are amino-oxy derivatives of polymer, preferably water-soluble polymers such as PEG, i.e., polyethylene glycol. The reagents of the subject invention may be used to covalently attach a variety of polymers to target macromolecules of interest.

An important advantage of the subject invention, particularly in the case of biological important macromolecules such as polypeptides, is that target

macromolecule can be modified by the attachment of water-soluble polymers without substantially reducing the biological activity of the macromolecule, or reducing the biological activity to a lesser extent than the biological activity would be reduced by the attachment of the same water-soluble polymers to multiple residues located throughout the macromolecule (typically lysine residues for a polypeptide) using activated polymers other than the site-specifically acting compounds of the present invention.

The term "biological activity" includes enzymatic activity, the ability to bind to receptors (including antibodies), the ability to bind ligands, the ability to induce an immune response, and the like.

A "complementary functional reactive group" is defined as one of a pair of functional groups that chemospecifically reacts with the complementary member of the pair. For example, amino-oxy-acetyl ("AoA") and glyoxylyl ("GXL") are complementary functional reactive groups that react to form an oxime bond.

The term "chemoselectively ligated" indicates the specific ligation that occurs between complementary orthogonal chemically reactive groups.

Complementary functional groups used in the invention are groups which can be used without compromising other groups present or other chemistry to be applied. Thus, by way of example, in the context of this application, amino-oxy-acetyl groups are a most preferred functional group.

Of course, it is apparent that the functionalized polymers may be used for attachment non-site-specifically to appropriately modified target molecules, for example, when multiple residues of a target polypeptide are first modified so that each contains a functional group specifically and complementarily reactive with the functional group on the polymers of the invention.

The functionalized polymer is attached to the target macromolecule site-specifically by covalent conjugation via an oxime linkage. "Covalently conjugated" or "conjugated" refers to the attachment of polymer to target via a functionalized polymer. "Functional" or "functionalized" describes, for the purposes of this invention, the attachment of a oxime-forming reactive group onto a polymer so that the polymer can be site-specifically conjugated to a complementary

functionalized target macromolecule. Generally, the PEG or POG (i.e., polyoxyethylated glycerol) molecule is activated or functionalized by attaching the reactive group to a terminal hydroxyl groups and then the functionalized polymer is covalently conjugated to an aldehyde group that has been site-specifically introduced into the target molecule. In sharp contrast to typical previous methods of conjugations where conjugation may occur between any reactive amino acids on the protein, the reactive amino acid is typically lysine, and lysine is linked to the reactive group on PEG or POG through its free ϵ-amino group, the functionalized polymer reagents of the invention are site-specifically attached via a stable oxime linkage to a single, pre-chosen, site on the target molecule.

A "target macromolecule" as used herein refers to an organic molecule (which includes molecules of biologic origin as well as organic molecules with inorganic components) having a molecular weight of at least 500, more preferably of at least 2000, even more preferably at least 5000, most preferably at least 10,000. A "functionalized target macromolecule" is a target macromolecule that has been modified site-specifically to contain an oxime-forming functional group. The target macromolecule can be derived from natural, recombinant sources or can be synthetic.

The term water-soluble functionalized polymer reagent as used herein refers to a water-soluble polymer modified so as to contain a functional group that provides for the site-specific conjugation of the water-soluble polymer to a target macromolecule through an oxime linkage. By the methods of the invention, target macromolecules are site-specifically modified to contain a functional group that is complementary in oxime-forming reactivity with the amino-oxy or aldehyde functional group introduced onto the polymer, preferably at one of its termini (or in the case of a bi-functional polymer--at both of its termini). By providing for water-soluble polymer reagents that can be coupled site-specifically to a polypeptide at an amino acid residue, preferably at the C- or N- terminus, it becomes possible to conjugate water-soluble polymers to proteins without substantially adversely affecting the biological activity of proteins that would be adversely affected through multiple couplings at amino acid residues located throughout the protein.

A "homogeneous" polymer conjugate composition of the invention refers to a chemical composition in which substantially all of the conjugate molecules have essentially identical chemical structures--each have the identical number and location of attached polymers although there can be a range in the molecular weight of the attached polymer(s). The molecular weight range average is typically that as found in commercially available preparations of a polymer used herein for functionalization; preferably the range is that obtained after further size fractionization of commerically available preparation. This is in sharp contrast to a typical polymer conjugate composition in which the individual molecules differ at least in the locations and in the number of attached polymers. Compositions of the invention can also be referred to as "self-identical" compositions, as substantially all of the individual molecules of the polymer conjugate are essentially identical to each other. Here "substantially all" refers to at least 80% of the total conjugate molecules all having the same location and number of polymers attached although each attached polymer may not be of the same size. Increasing degrees of purity, such as 90%, 95%, 98%, 99%, 99.5%, 99.8%, etc., all the way to 100%, are increasingly preferred meanings of "substantially all."

As will be readily appreciated in light of the present invention, homogeneous polymer conjugate compositions can be comprised of homo- or hetero-polymic, wherein hetero-polymers as defined herein refers to differences in the chemical type or in the MW(av) of polymers bound, each of which can be present at its molecular weight average. As discussed in the remainder of the application, when a heterobi- or heteromulti-polymer functionalized-polymer is attached to a target macromolecule not all of the polymer molecules attached at the single site will be identical, nevertheless the composition can be homogeneous as defined herein.

In one embodiment of the invention the formula of the compounds useful for coupling polymers to complementary functionalized target macromolecules, such as polypeptides, via an oxime linkage is:

$$P\text{-}X\text{-}O\text{-}NH_2$$

Where P represents an organic polymer, preferably water soluble, X represents a spacer group, $-O-NH_2$ represents amino-oxy. Water-soluble organic

polymers of interest preferably have hydroxy groups appended to the polymer backbone that are convenient sites for functionalization and may be selected from known water-soluble polymers including but not limited to: (a) dextran and dextran derivatives, including dextran sulfate, P-amino cross linked dextrin, and carboxymethyl dextrin, (b) cellulose and cellulose derivatives, including methylcellulose and carboxymethyl cellulose, (c) starch and dextrines, and derivatives and hydroylactes of starch, (d) polyalklyene glycol and derivatives thereof, including polyethylene glycol, methoxypolyethylene glycol, polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, (e) heparin and fragments of heparin, (f) polyvinyl alcohol and polyvinyl ethyl ethers, (g) polyvinylpyrrolidone, (h) α,β-Poly [(2-hydroxyethyl)-DL-aspartamide, (i) polyoxyethylated polyols, and (j) polynucleotides, including deoxyribonucleotides, ribonucleotides, and their phosphate-backbone-modified derivatives, e.g phosphorothioate derivatives. Preferred polynucleotides are those oligonucleotides of homology to biologically important polypeptides, preferably of viral or mammalian origin, and can include antisense sequences.

Water-soluble polymer reagents include but are not restricted to polypropylene glycol ("PPG"), polyoxyethylated polyol ("POP"), heparin, heparin fragments, dextran, polysaccharides, polyamino acids including proline, polyvinyl alcohol ("PVA"), and other water-soluble organic polymers. The water-soluble polymer reagents of the subject invention include amino-oxy derivatives of polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, polyvinyl ethyl ethers, and α,β-Poly [(2-hydroxyethyl)-DL-aspartamide] and other water-soluble organic polymers. U.S. Pat. No. 4,179337; U.S. Pat. No. 4,609,546; U.S. Pat. No. 4,261,973; U.S. Pat. No. 4,055,635; U.S. Pat. No. 3,960,830; U.S. Pat. No. 4,415,665; U.S. Pat. No. 4,412,989; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,414,147; U.S. Pat. No. 3,788,948; U.S. Pat. No. 4,732,863; U.S. Pat. No.

4,745,180; EP No. 152,847; EP No. 98,110 published January 11, 1984; JP No. 5,792,435, describe various polymers that may be functionalized according to the invention, and are hereby incorporated by reference. Preferably, the water-soluble polymer P is selected from dextran and dextran derivatives, dextrine and dextrine derivatives, polyethylene glycol and derivatives thereof. Most preferably, the water-soluble polymer P is selected from polyethylene glycol and derivatives thereof, the monomethyl ether of polyethylene glycol being particularly preferred (so as to avoid cross-linking between proteins). Water soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol ("POG"), etc. POG is preferred. The glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al. (1988) J. Biol. Chem., 263:15064-15070, and can generally substitute for PEG or its derivatives in the formulas herein.

When polypeptides modified by the water-soluble polymer reagents of the subject invention are to be used as pharmaceuticals, polymer P should be non-toxic, and preferably non-immunogenic.

As the polymer P comprises multiple repeating units of varying amounts, it will be appreciated that the molecular weight of P may vary considerably. Furthermore, when P is said to have a given molecular weight, that molecular weight may only be approximate, reflecting the average molecular weight of a population of molecules P differing with respect to one another in regards to the number of subunits present in the molecule. In general, P will have a molecular weight of about 200 to 200,000, preferably in the range of about 400 to 50,000, more preferably 1,000 to 60,000, even more preferably in the range of 2,000 to 40,000. Suitable molecular weights for P, when the functionalized polymer are to be coupled to a polypeptide will vary in accordance with the specific protein to modified and the purpose of the modification.

The spacer group X is optionally present. This invention provides functionalized polymers in which the complementary reactive group capable of oxime-linkage formation is an amino-oxy group. Preferred is amino-oxy-acetyl ("AoA"). In fact, additional structure (X or spacer group) can be used which connects an amino-oxy group to the polymer of interest. The spacer group X represents a non-reacting group comprising substituted or unsubstituted, branched or linear, aliphatic or aromatic groups such as phenyl or C_1-C_{10} alkylene moieties, C_1-C_{10} alkyl groups, or a combination thereof, or an amino acid chain (such as a flexible hinge or loop sequence (see for example Argos, J. Mol. Biol. (1990) 211:943-958), or a nucleotide chain or a sugar chain or a lipid chain or a combination thereof and may contain heteroatoms. In the preferred embodiments of P-X-ONH_2, X comprises -CH_2- or -CHOH-, or more preferably -CO-CH_2- or -NH-CO-CH_2-. For example, in Example 1 below, X is -NH-CO-CH_2- in the formula for AoA-MPEG: CH_3-O-(CH_2CH_2O)nCH_2CH_2-NH-CO-CH_2-O-NH_2. When an amino-oxy group is on the functionalized polymer groups present in the additional connecting structure (spacer groups) adjacent to the amino-oxy function are not critical; however, a requirement of these spacer groups is that they do not interfere with the formation of the oxime linkage between the amino-oxy and its complementary aldehyde group. They should not react in preference to the amino-oxy group with the aldehyde, nor provide steric hindrance to the reaction, nor deactivate the reactive groups. Where the conjugated polymer is to be used for antigenic or immunogenic purposes, it is apparent to one skilled in the art that spacer groups are chosen that are not themselves strongly immunogenic. Where the conjugated polymer is to be used for binding purposes, the preferred spacer group enhances or at least does not interfere with properties such as binding, avidity, product stability or solubility.

The spacer group X can be chosen to enhance hydrolytic stability of the oxime linkage. The hydrolytic stability of oximes is influenced by their structure; data indicate that oxime stability increases in the series:
-CO-NH-CH_2-CH=N-O-CH_2- $\leq$ -CO-NH-CH_2-C(R)=N-O-CH_2- $<$
-NH-CO-CH=N-O-CH_2- $<$ -C_6H_4-CH=N-O-CH_2-. Increased oxime stability may be obtained with the presence of aromatic groups in X.

In a further embodiment of the invention the functionalized polymers are bi-functional, i.e. will have two oxime-forming groups. The groups are not complementary in reactivity to each other. Preferably two groups are at distal, non-interfering (with each others ability to form an oxime bond) sites on the polymer, more preferably at each of termini. For example, dihydroxy polyalkylene glycols are functionalized as taught herein to obtain a polymer having an amino-oxy oxime-forming group at each of its termini. The bis-aminooxy polymer is reacted with the site-specifically functionalized target macromolecules of the invention to obtain a "dumbbell" construction where the polymer through oxime linkages acts to link a first functionalized target macromolecule and second functionalized target macromolecule, preferably identical to the first, through each of their site-specific oxime-forming groups. Hetero-dimers can be formed when the first and second macromolecules are different. Hetero-dimers can be formed, for example, by sequential reactions of the first and second functionalized macromolecule (for example, by the intermediate step of isolating a polymer conjugate wherein only one of the two polymer reactive groups are linked to a first target macromolecule) or by a single reaction of the polymer with a mixture of the first and second functionalized macromolecule present in the same molar ratio. The dumbbell constructs of the present invention, and compositions thereof, have the target macromolecules, preferably biologically important polypeptides, linked site-specifically, thus providing constructs, in contrast to previous polymer conjugation methods, of defined structure and homogeneous composition. Preferred embodiments of the bi-functional polymer contain two amino-oxy acetyl (AoA) groups. Particularly preferred is AoA-PEG-AoA, where PEG or its derivatives comprise the polymer moiety.

In additional embodiments of the invention the oxygen atom of the amino-oxy group can be replaced with a sulphur atom, in which case chemoselective ligation with an aldehyde or ketone functional group on a target macromolecule would yield a thio-oxime bond -C(R)=N-S-.

In another embodiment of the invention bi- and multi-polymer-containing functionalized polymers are provided. Functionalized polymers of these embodiments have the general formulas

$$(P)_mL\text{-}X\text{-}O\text{-}NH_2$$

or

$$(P)_mL\text{-}X\text{-}C(R)O$$

where P is an organic polymer as defined herein, m is an integer from 2 to 10, more preferably 2 to 5, X is a spacer group as defined herein, -O-NH_2 is amino-oxy, -C(R)O is aldehyde when R is hydrogen, and L is a multi-valent linking group to which each P (m in number) is separately and covalently linked, and wherein the valency of L is at least m+1. When -C(R)O is a ketone, R is preferably C1-C10, more preferably C1 to C4, linear or branched alkyl group. In the aldehyde or ketone functionalized polymer, groups present in the X spacer adjacent to the aldehyde or ketone function are not critical; however, a requirement of these groups is that they do not interfere with the formation of the oxime linkage between the aldehyde and its complementary amino-oxy group. They should not react in preference to the aldehyde group with the amino-oxy, nor provide steric hindrance to the reaction, nor deactivate the reactive groups. The connecting group does not react with other functions present but if designed to do so then does not do so in an undesirable way (i.e., a way which reduces product homogeneity or activity). The spacer group preferably represents a non-reacting group comprising substituted or unsubstituted aliphatic or aromatic groups such as phenyl or C_1-C_{10} alkylene moieties, C_1-C_{10} alkyl groups, or a combination thereof, or an amino acid chain (such as a flexible hinge or loop sequence (see for example Argos, J. Mol. Biol. (1990) 211:943-958), or a nucleotide chain or a sugar chain or a lipid chain or a combination thereof and may contain heteroatoms. Preferably when the oxime-forming group comprises -X-C(R)O, X is a spacer group comprising -CH_2-, -CO-, or -CHOH, more preferably -C_6H_4-. Also preferred are an aldehyde and spacer comprising OHC-CO- or glyoxylyl ("GXL").

In additional embodiments of the invention the oxygen atom of the amino-oxy group can be replaced with a sulphur atom, in which case reaction with an aldehyde or ketone functional group would yield a thio-oxime bond -C(R)=N-S-.

In the case of bi-and multi-polymer functionalized polymers of the invention, conjugation of the functionalized polymer site-specifically to a target macromolecule results in attachment of two or more polymers site-specifically through a single oxime linkage via the -O-NH_2 or -CHO group attached to the multi-valent L structure. Amino-oxy-functionalized polymers are preferred over the aldehyde embodiments in part because of the methods available and those taught herein to introduce an aldehyde (or keto) function on a target macromolecular.

Accordingly, the bi- or multi-polymer-containing functionalized polymer enables two or more polymers, the same or different, preferably the same, to be attached to a single, pre-chosen site on the target macromolecule. Where L is a trivalent group, n is 2 (see Example 2 or 10 herein). Preferably the valence of L is m+1, wherein one valency of L is occupied by the oxime-forming group optionally through X, and the remaining valencies of L are occupied by one or more, i.e. m, polymers. The structure of L is not critical nor are the linkages connecting L to the polymers so long as L provides no steric hindrance to the subsequent oxime reaction, nor deactivate the reactive groups. L does not react with other functions present. Each arm or valency of the linking group L in the functionalized polymer preferably comprises a non-reacting group comprising substituted or unsubstituted aliphatic or aromatic groups such as phenyl or C_1-C_{10} alkylene moieties, C_1-C_{10} alkyl groups, or a combination thereof, or an amino acid chain (such as a flexible hinge or loop sequence (see for example Argos, J. Mol. Biol. (1990) 211:943-958), or a nucleotide chain or a sugar chain or a lipid chain or a combination thereof and may contain heteroatoms. Prior to conjugation with a polymer, preferably all but one arm or valency of L contains a functional group that can react specifically with a group on the polymer, which preferably is located at a polymer terminus. The remaining valency is protected for later reaction with, or otherwise occupied with, a compound providing the oxime-forming function (said function is in a deprotectable state if desired). Where the polymer conjugate is to be used for antigenic or immunogenic purposes, it is apparent to one skilled in the art that linker groups are chosen that are not themselves strongly immunogenic. Where the polymer conjugate is to be used for binding purposes, the preferred linker group enhances or at least does not interfere with properties such as binding, avidity, product stability

or solubility. Linking structures can themselves contain valencies occupied with oxime-forming groups such that parallel assembly via oxime formation with a complementary functionalized polymer of the invention is employed to assemble the $(P)_mL$- structure. Accordingly, baseplate structures described in co-pending United States Serial Numbers 08/057,594, 08/114,877, and 08/057,594, and co-pending International application PCT/IB94/00093 (which are hereby incorporated by reference) are suitable for use as L structures. The oxime-forming groups of the baseplates can be replaced with other complementary reactive groups; however, most preferably oxime formation is used for assembly. For an example where non-oxime chemistry is used for $(P)_mL$- assembly, see Example 4 herein where L is pentalysyl peptide (where m is 5) with each lysyl residue containing a covalently attached polymer and the peptide N-terminal contains the oxime-forming group. A preferred L structure is derived from a tri-amine compound wherein any two amino groups are each available for coupling to a polymer and the remaining amino group is available for introduction of an oxime-forming group. A preferred tri-amine is a compound of the formula $N(R5\text{-}NH_2)_3$, wherein any two amino groups ($-NH_2$) are available for coupling to the polymer and the remaining amino group is available for introduction of an oxime-forming group, and R5 is a non-reacting group comprising substituted or unsubstituted aliphatic or aromatic groups such as phenyl or C_1-C_{10} alkylene moieties, C_1-C_{10} alkyl groups, or a combination thereof, or an amino acid chain (such as a flexible hinge or loop sequence (see for example Argos, J. Mol. Biol. (1990) 211:943-958), or a nucleotide chain or a sugar chain or a lipid chain or a combination thereof and may contain heteroatoms. R5 is preferably -CH2-CH2-. The three primary amino groups are preferably distal to the nitrogen. Most preferably the tri-amine compound is tris-(2-aminoethyl)amine.

As is taught herein a great flexibility is available to one in the art for designing and obtaining L-structures of desired sequence, structure, valency, and function with specifically placed complementary reactive groups for attachment of polymers. Additional methods and compounds, which were developed in the laboratories of the present inventors and which are suitable for L-structure synthesis, are described in Rose (*J. Amer. Chem. Soc.* "Facile synthesis of artificial proteins" (1994) 116:30-33; incorporated herein by reference).

As discussed herein, bi- and multi-polymer functionalized polymers of the invention find particular use in systematically modifying the Stokes radius of a target macromolecule, which in turn, preferably modifies a particularly desirable property of that macromolecule. In the case of pharmaceutically important polypeptides, the Stokes radius can be systematically modified, which in turn preferably allows systematic modification of its pharmacokinetic behavior, and ultimately its therapeutic efficacy.

In preferred embodiments of the invention, the functionalized polymers contain PEG or its derivatives as the polymer backbone. In one embodiment the functionalized polymer is a mono-functional or a bi-functional polymer that contains PEG or a derivative thereof and has the structure

R1-O(R2-O)nR2-R3

where n is an integer between 5 and 2,000, R2 is a lower alkyl group which is straight, branched, substituted, or unsubstituted, and (a) one of R1 and R3 comprises an amino-oxy oxime-forming group and an optional spacer group and the other of R1 and R3 is hydrogen, -CH3, or a protective group, or (b) both R1 and R3 comprise an amino-oxy oxime-forming group and an optional spacer group. The R2 of each repeating unit in the polymer can be independently the same as or different from each other. The number of repeating units, defined by n, is between 5 and 2000, preferably between 10 and 1,000, and more preferably between 50 and 800. Accordingly, the polymer has an average molecular weight between about 200 and 100,000, preferably between about 400 and 50,000, and more preferably between about 2000 and 40,000. Typically the molecular weight (average) of starting PEG is that available from (but not limited by) commercial sources and includes the group consisting of 5000, 10,000, and 20,000. R1 or R3 contains a functional amino-oxy group capable of forming an oxime linkage with a complementary aldehyde or ketone functional group on a target second organic macromolecule. The oxime-forming group comprises -O-NH_2. Preferably the oxime-forming group comprises -X-O-NH_2 where X is a spacer group defined as herein. Preferably when the oxime-forming group comprises -X-O-NH_2, X is a spacer group comprising -NH-CO-R4- where R4 is a linear, branched, or cyclic lower alkyl, substituted or unsubstituted, preferably CH_2, and where R4 is directly

attached to -O-NH_2. A protective group is non-reactive with respect to the polymer functionalization methods of the invention or the target macromolecules of the invention. Preferably the protective group has between 1 and 10 carbons, more preferably it is an alkyl group, most preferably it is methyl. Prior to functionalization, preferably PEG has at least one hydroxy group more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated (functionalized) to introduce a functional group capable of site-specifically forming an oxime linkage with a complementary reactive group introduced site-specifically on a target macromolecule. By "lower alkyl" group is meant a C1 to C10, preferably C2 to C4, alkyl group.

In another preferred embodiment of the invention, bi- and multi-polymer-containing functionalized polymers containing PEG or its derivatives are provided. These embodiments are those bi- and multi-polymer functionalized polymers as discussed above, but wherein two or more PEG polymers are attached to a single oxime forming group through individual attachment to the linking structure. The bi- and multi-PEG functionalized polymers of the invention find particular use in systematically modifying the Stokes radius of a polypeptide, which, in the case of pharmaceutically important polypeptides, preferably allows systematic modification of its pharmacokinetic behavior, and ultimately its therapeutic efficacy.

More specifically, the invention relates to preparation and use of a functionalized PEG and dextran: monofunctional MPEG-NH-CO-CH2-O-NH_2, Dextran-O-CH(CHOH-CH_2OH)-$(CHOH)_2$-CH_2-NH-$(CH_2)_2$-NH-CO-CH_2-ONH_2, Dextran-O-CH(CHOH-CH_2OH)-$(CHOH)_2$-CH_2-NH-$(CH_2)_2$-NH-CO-C_6H_4-CHO, bifunctional NH_2-O-CH2-CO-NH-PEG-NH-CO-CH_2-O-NH_2, and multi-PEGylated $(PEG)_2$Lys-NH-$(CH_2)_2$-NH-CO-CH_2-O-NH_2, HO-$((PEG)Lys)_5$-NH-CO-CH_2-O-NH_2, and (PEG-aminoethyl)$_2$-N-$(CH_2)_2$-NH-CO-CH_2-O-NH_2.

In a preferred embodiment of the invention, functionalized polymers, as taught herein, containing dextran or its derivatives as the polymer backbone are provided. In another preferred embodiment the functionalized polymer is a mono-functional or a bi-functional polymer that contains dextran or a derivative thereof. In yet another preferred embodiment, bi- and multi-polymer functionalized polymers, as taught herein, containing dextran or its derivatives are provided.

These embodiments are those bi- and multi-polymer functionalized polymers as discussed above, but wherein two or more dextran polymers are attached to a single oxime forming group through individual attachment to the linking structure.

In another embodiment of the invention methods are provided for producing the functionalized polymers of the invention. In the general case, a polymer as described above having at least one, preferably terminal, reactive group is modified at that reactive group to contain an oxime-forming group. Any of numerous modifying chemistries can be used depending on the reactive group available on the polymer. For example, in the preferred case of PEG or its derivatives, a terminal hydroxyl group is available. Should more than one reactive group be present, a form of the polymer in which all but one reactive group (or all but two when a bi-functional polymer is desired) is protected from reaction can be used a starting material. Modification to an oxime-forming group can occur in sequential steps when convenient. For example, the reactive group on the polymer can be converted to another reactive group that is readily acylated with a protected-amino-oxy-containing compound such as Boc-NH-O-CH2-COOSu. The amino-oxy group can then be deprotected before use or before storage. The most reactive group towards acylating agents is $-NH_2$. A preferred PEG intermediate is CH3-O-(CH2-CH2-O)n-CH2-CH2-NH_2, or when a bifunctional polymer is desired, H2N-(CH2-CH2-O)n-CH2-CH2-NH_2. MPEG can be readily converted to CH3-O-(CH2-CH2-O)n-CH2-COOH by the steps of carboxymethylation (Royer and Ananthanmaiah (1979) J. Am. Chem. Soc. 101:3394-3396).

Using the above intermediate PEG-NH_2 compound, an oxime-forming group is introduced at each amino function by acylation with BOC-NH-O-CH2-COOSU. As shown in Example 1, PEG-NH_2 was prepared via the following series of reactions PEG-OH → PEG-Cl → PEG → N_3 → PEG-NH_2 according to a procedure already described (Zalipsky et al., (1983), Eur. Polym. J.. 19, 1177-1183).

In another embodiment, bi- or multi-polymer functionalized polymers are synthesized by first obtaining an L structure of desired multi-valency, usually having one valency protected, and then reacting the protected L-structure with an appropriately activated polymer intermediate typically using linking chemistries

known in the art or with a functionalized polymer of the invention via oxime chemistry. After isolation of the bi- or multi-polymer product, the product is functionalized according to the invention by deprotection of the protected remaining valency of L followed by subsequent reaction, e.g. acylation in the case of an amino group, with a suitably protected-amino-oxy or protected-aldehyde containing acylating group. After deprotection of the oxime-forming functional group, the final product, the bi- or multi-polymer functionalized polymer is obtained.

Alternatively, L is first derivatized with a suitably protected-amino-oxy or protected-aldehyde containing group, the mono-substituted derivative is isolated, the mono-substituted L derivative is then reacted at each remaining valency with a polymer intermediate (such as one having a COOH when L contains NH_2 or an NH_2 group when L contains COOH) or with a functionalized polymer of the invention (when oxime chemistry is used to assemble P to L). For example, MPEG-COOH intermediate polymers, which can react with free amino groups on an L-structure, are provided. The coupling will take place in the presence of HOBt and DCC, or without these reagents if the succinimidyl derivative of MPEG-COOH was previously prepared. After deprotection of the oxime-forming functional group, the final product, the bi- or multi-polymer functionalized polymer is obtained.

When the L group is formed from amino acids, the peptide sequence of an L structure can be synthesized by routine solid phase peptide synthesis ("SPPS"), and while the peptide is still attached to the solid phase PEG-COOH in an activated form, such as the N-hydroxysuccinimide ester, can be added to the nascent peptide chain. For example, the L structure can consist of a peptide having six reactive groups such as five lysine residues and an N-terminal amine group. PEG-COOSu hydroxysuccinimide ester can react with each of the ϵ-amino groups of the lysine residues (while the N-terminus α-amino group is left protected). The N-terminal amine group of the fully acylated peptide is then unprotected and the polymer-containing structure is reacted with a Boc-AoA-containing active ester to introduce the AoA group, which after Boc removal and mild cleavage from the resin, yields a penta-polymer-containing functionalized polymer of the invention. It is noteworthy that this method finds particular use with synthetic structures (and perhaps certain recombinant products) since these can be designed to exclude additional residues,

e.g., amino acid residues, that would require protection during the process and deprotection afterwards. Alternatively, Boc-Ser(benzyl)-OH or Boc-Ser(t-butyl)-OH in an activated form, such as the N-hydroxysuccinimide ester, can be attached tot he ϵ-amino groups of the lysine residues. The N-terminus α-amino is then deprotected to introduce an amino-oxy group, e.g. AoA, so that after Boc removal a precursor L structure containing ϵ-Ser-pentalysine is obtained. Treatment of the precursor L structure once conjugated to the protein with a mild oxidizing agent, such as periodate at Ph 7, will convert ϵ-Ser-pentalysine to ϵ-GXL-pentalysine, thus producing a penta-GXL L structure that can then be reacted with an amino-oxy functionalized polymer of the invention. The oxidation reaction can be terminated using any 1,2-diol or 1-amino-2-ol or 1-ol-2-amino compound having relatively free rotation about the 1,2 bond, such as ethylene glycol. Alternatively, the oxidation reaction can be terminated by rapid removal of periodate, for example by reverse phase high performance liquid chromatography (RP-HPLC). Since the oxidation reaction only occurs with serine residues containing a primary amino group, only the ϵ-serine residues are converted to the glyoxylyl. One skilled in the art knows of methods for chemically protecting an N-terminal serine from oxidation, when such protection is desired. The N-terminal amine group can then be unprotected and the polymer-containing structure is reacted with a Boc-AoA-containing active ester to introduce the AoA group, which after Boc removal, yields a penta-polymer functionalized polymer of the invention. BOC or the typical amino-protecting groups used in peptide synthesis that can subsequently be removed under mild conditions relative to the product are suitable (see for example Green and Wuts (1991) "Protective groups in organic synthesis," 2nd ed., Wiley, New York, NY).

Functionalized target macromolecules can be designed and prepared to site-specifically introduce an oxime-forming group using methods known in the art, including methods found in co-pending United States Serial Numbers 08/057,594, 08/114,877, and 08/057,594, 07/869,061, and 08/241,697, and co-pending International application PCT/IB94/00093 (which are hereby incorporated by reference). Target macromolecules can be obtained by recombinant methods or isolated from natural sources, and an oxime-forming complementary reactive group,

an aldehyde or amino-oxy group, is site-specifically formed at a desired location of the macromolecule.

In a preferred embodiment of the present invention functionalized polymers are prepared that are reactive site-specifically with polypeptide compounds, or with compounds or materials containing amino acids, in which an amino acid has been modified to contain an oxime-forming group specifically reactive with the oxime-forming function introduced onto the polymer. Preferably the N-terminal or C-terminal residue of a polypeptide is so modified using methods presented herein. Co-pending applications United States Serial Numbers 08/114,877, filed August 31, 1993; 08/105,904, filed May 12, 1994, as well as PCT/IB94/0093, filed May 5, 1994, each of whose contents are hereby incorporated by reference, present additional methods for site-specific modification of target compounds, particularly polypeptides by both chemical and enzymatic means, that find use with the functionalized polymer compounds of the present invention and methods for their conjugation to target molecules as taught herein.

In the case of a polypeptide, the oxime-forming group is introduced preferably at a C-terminal of the polypeptide by selective enzyme catalyzed reverse proteolysis or at an N-terminal serine or threonine by mild oxidation. (See for example Geoghegan et al. (Bioconjugate Chem. (1992) 3:138-146); Gaertner et al. (Bioconjugate Chem. (1992) 3:262-268); EP 243929; and WO90/02135, which are incorporated herein by reference.) A recombinant or natural peptide may have multiple C- or N-termini, such as would occur in a dimer or tetramer, each of which can be functionalized.

The water-soluble polymer reagents of the invention may be used to modify a variety of polypeptides or similar molecules that have been site-specifically modified to contain a complementary functional group capable of oxime formation with the functional group on the polymer. Polypeptides of interest include: antibodies, monoclonal and polyclonal; cytokines, including, M-CSF, GM-CSF, G-CSF, stem-cell growth factor; lymphokines, IL-2, IL-3, growth factors, including, PDGF, EGF; peptide hormones, including, hGH, erythropoietin; blood clotting factors, including, Factor VIII; immunogens; enzymes; enzyme inhibitors; ligands and the like. Polypeptides of interest for water-soluble polymer derivatization by

the water-soluble polymer amino-oxy or aldehydes include hormones, lymphokines, cytokines, growth factors, enzymes, vaccine antigens, and antibodies. Water-soluble polymer derivatization of erythropoietin (EPO), especially human erythropoietin is of particular interest. Polypeptides of interest may be isolated from their natural sources, genetically engineered cells, or produced by various in vitro synthesis methods. The following patent applications (which are hereby incorporated by reference) report PEGylated modifications of various biologically important proteins: U.S. Pat. No. 4,179337; U.S. Pat. No. 4,609,546; U.S. Pat. No. 4,261,973; U.S. Pat. No. 4,055,635; U.S. Pat. No. 3,960,830; U.S. Pat. No. 4,415,665; U.S. Pat. No. 4,412,989; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,414,147; U.S. Pat. No. 3,788,948; U.S. Pat. No. 4,732,863; U.S. Pat. No. 4,745,180; EP No. 152,847; EP98110 published January 11, 1984; JP5792435. The proteins, in their unmodified state, are target macromolecules for site-specific functionalization and subsequent site-specific polymer-conjugation, as taught herein.

A peptide, polypeptide, or protein (used interchangeably herein) shall mean both naturally occurring and recombinant forms, as well as other non-naturally occurring forms of the peptide or protein which are sufficiently identical to the naturally occurring peptide or protein to allow possession of similar biological or chemical activity. As is known in the art peptides can be formed from of non-naturally occurring or non-proteinogenic amino acid residues. Furthermore, as is well known in the art, amino acid residues can be joined via non-amide linkages. Peptides or proteins can also contain protecting groups at either terminal that prevent or minimize degradation of the peptide or protein in vivo.

While the water-soluble polymer reagents of the subject invention may be used to modify most polypeptides, it is of particular interest to modify (1) polypeptides for use as drugs, and (2) polypeptides for use in assays. Polypeptide for use in assays include specific binding proteins, polypeptides recognized by specific-binding proteins, and enzymes. By specific-binding proteins it is intended antibodies, hormone receptors, lectins, and the like. By the term "antibodies," it is intended to include both polyclonal and monoclonal antibodies with natural immunoglobulin sequences, synthetic antibody derivatives, and the like; antibodies may be modified so as to be joined to any of a variety of labels, fluorescent,

radioactive, enzymatic, biotin/avidin or the like. Synthetic antibody derivatives include natural immunoglobulin sequences that have been mutated and selected for altered binding specificity, various immunoglobulin gene derived polypeptides, typically single chain, produced by genetically modified bacteria, antibodies modified so as to contain modified constant regions and the like; a review of such synthetic antibody derivatives based on the principles of antibody formation is provided in Winter and Milstein, Nature, 349:293-299 (1991). An antibody is a glycoprotein of the globulin type that is formed in an animal organism in response to the administration of an antigen and that is capable of combining specifically with the antigen. These are also referred to as immunoglobulins. Antibody fragments can retain some ability to selectively bind with their antigen or hapten. The ability to bind with an antigen or hapten is determined by antigen-binding assays (see, for example, *Antibodies: A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor, New York (1988), which is incorporated herein by reference). Such antibody fragments include, but are not limited to, Fab, Fab' and $(Fab')_2$. A native antibody is one which is isolated from an animal or from an animal or hybrid animal (hybridoma) cell line.

The teachings herein describing groups present in the additional connecting structure (spacer group) adjacent to the aldehyde function or the amino-oxy function present on a functionalized polymer applies to any spacer group present on functionalized target molecules as well.

In one embodiment of the invention, the C-terminus is modified, preferably with enzymes that can direct bifunctional reagents with suitable reactive groups specifically at the C-terminus in polypeptides or proteins (e.g. antibodies). The carboxyl terminus of a polypeptide chain is, at least in terms of primary structure, in most cases far from the active site of a protein. In another embodiment of the invention, use is made of the fact that specific bifunctional reagents with suitable reactive groups preferably or specifically react at non-carboxy terminus sites of a polypeptide molecule. In a most preferred embodiment, the amino terminal group is the site-specific target after reaction to activate the N-terminal site on a peptide. In a preferred reaction, N-terminal serine ("Ser") and threonine ("Thr") residues are oxidized in an exceedingly mild reaction with periodate (e.g. 20°C, 26 μM protein,

10mM imidazole buffer pH 6.95, 2-fold excess of periodate for 5 min). N-terminal Ser reacts about 1000 times as fast as other protein groups (Fields and Dixon, (1968) Biochem. J. 108:883), so that a high degree of specificity is obtained. For greater generality, N-terminal Ser or Thr can be introduced by recombinant DNA techniques, or, in appropriate cases, by selecting a source of the protein of interest which has a natural Ser or Thr N-terminus, or by enzymatic cleavage, with for example an aminopeptidase, dipeptidylpeptidase, or proline specific endopeptidase, of non-essential terminal amino acids to expose a Ser or Thr. A second preferred reaction is the use of transamination to convert an N-terminal residue of a protein into an active carbonyl function, preferably by reaction with glyoxylate. This reaction proceeds under relatively mild conditions (see e.g. Dixon and Fields, (1979) Methods in Enzymology, 25:409-419). A wide range of N-terminal residues can be transaminated. Besides the alanine, asparagine, glutamine acid and phenylalanine of the proteins mentioned in the preceding reference (Methods in Enzymol., 25:409-419), methionine was transaminated in the specific case of Antril, as described in Example 8. The polypeptidyl N-terminal aliphatic aldehydes or ketones produced by these techniques may be reacted with polymers preferably functionalized with amino-oxy-acetyl groups.

A protected aminooxyacetyl group was directly attached to polymers already functionalized with either a unique amino group as it was the case with methoxy polyethylene glycol (Example 1) or dextran (Example 5), or two amino groups as in case of H_2N-PEG-NH_2 (Example 2) for the construction of the dumbbell or to a polyfunctionalized linker, in order to synthesize a multibranched structure which is conjugated to the protein at a single site (Examples 3, 4, 10). Described structures are the following:

$(PEG\text{-}CONH\text{-}(CH_2)_2)_2N\text{-}(CH_2)_2\text{-}NHCO\text{-}CH_2\text{-}O\text{-}NH_2$

$PEG\text{-}Lys(PEG)\text{-}NH\text{-}(CH_2)_2\text{-}NHCO\text{-}CH_2\text{-}O\text{-}NH_2$

$H_2N\text{-}O\text{-}CH_2\text{-}CO\text{-}(Lys(PEG))_5$

Since a small bivalent aminooxy tag can also be attached site-specifically at a single site to a protein (Example 14), the polymer to be conjugated has to be functionalized with the complementary aldehydic function, e.g. PEG-O-CH_2-CHO

or PEG-NHCO-C_6H_4-CHO, as described in Example 12, which can be obtained by reacting, for example, amino functionalized PEG with carboxy(benzaldehyde)OSu.

In the same way, a polyaminooxy tag can be introduced at a single site, in order to obtain a multiarm-conjugate, as it is shown in Example 18, with (H_2N-OCH_2-$CONH(CH_2)_2)_3N$ and PEG-CHO.

In less preferred embodiments, the site-specific target is a side-chain group of a polypeptide not necessarily non-terminal, preferably an amino acid residue present at a single copy or otherwise preferentially available and sensitive to modification. Alternatively, a unique residue may be introduced by recombinant methods. The side-chain group may be first modified using method taught herein to put in place a reactive group (aldehyde, keto or AoA) that will subsequently specifically react with a complementary reactive group on a functionalized polymer (e.g. AoA or aldehyde).

In another embodiment of the invention, a target macromolecule, particularly a polypeptide, is modified site-specifically at a location other than at its termini. Modifications to the primary structure itself, by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation, can be made without destroying the activity of the protein. Methods for making such modified proteins, known as "muteins", are described in U.S. Pat. No. 4,518,584 issued May 21, 1985, and U.S. Pat. No. 4,752,585, issued June 21, 1985, both are hereby incorporated by reference, and are well-known in the art (see Current Protocols in Molecular Biology, ed., Ausubel (1994), Greene Pub. Associates and Wiley-Interscience, J. Wiley, New York, NY; all volumes of which are hereby incorporated by reference). For example, at least one amino acid residue which is not essential to biological activity and is present in the biologically active protein can be replaced with another amino acid which is amenable to subsequent site-specific modification to create a functionalized target macromolecule of the invention.

Polymers functionalized with a hydrazide group can also react with the specifically introduced aldehydic or keto group, to form a hydrazone linkage. Though this bond is known to be less stable than the oxime bond, especially under

acidic conditions, it should be of interest in some special cases where the protein have to be released later in vivo.

Target macromolecule polypeptides can be produced by a prokaryotic microorganism or a eukaryotic cell that has been transformed with a native or modified polypeptide-encoding DNA sequence, preferably of human origin. Variants of naturally occurring polypeptides, wherein substantial identity of amino acid sequences has been maintained (i.e., the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) that do not cause a substantially adverse functional dissimilarity between the mutationally altered protein and native protein) are useful herein.

In another embodiment of the present invention are provided a target macromolecule modified by a polymer reagent molecule, i.e. the subject water-soluble functionalized polymer amino-oxy or aldehyde reagents, so as to be covalently bonded to one or more water-soluble polymers at a single site on the target macromolecule via an oxime linkage. These are referred to herein as "polymer conjugates." As discussed above, preferably the target molecule is a polypeptide, more preferably a polypeptide of biological importance.

In a preferred conjugate embodiment is provided a polypeptide derivatized with water-soluble polymer PEG or its derivatives via an oxime linkage.

The subject invention includes functionalized target macromolecules described above modified by reaction with the functionalized polymers molecules described above, wherein one or more polymers are covalently attached via a single, uniquely located, oxime linkage. In one embodiment these polymer conjugates are represented by formulas

B-C(R)=N-O-X-P or B-C(R)=N-O-X-L(P)$_m$ or B-O-N=C(R)-X-L(P)$_m$, wherein P is an organic polymer, preferably water soluble, as described herein, B represents a functionalized target macromolecule, preferably a polypeptide, as described herein, m represents an integer in the range 2 to 10, X and L and R are as defined herein, and -C(R)=N-O- is an oxime bond. R is preferably H. R can be the side chain of an N-terminal residue of a polypeptide which has been converted to an active carbonyl by transamination. Preferably m is the range of about 2 to 10, the range of 2 to 5 being particularly preferred. Preferably B is a polypeptide wherein

preferably P is covalently joined site-specifically to a functionalized N- or C-terminal residue on B. In additional embodiments of the invention the oxygen atom in the oxime bond of the formulas is replaced with a sulphur atom to obtain a -C(R)=N-S- thio-oxime bond.

Individual functionalized target macromolecules can be derivatized by one or more different water-soluble polymers by means of reaction with different embodiments of the polymer compounds of the invention. Individual target macromolecules can be modified with multiple water-soluble polymers at a single site when m is greater than one. Preferably P is a polyalkylene glycol or a dextran derivative.

Biological activities of proteins modified with PEG or dextran polymers are preserved to a large extent as shown by the Examples below.

Salts of any of the macromolecules described herein, e.g., polypeptides, water-soluble polymers and derivatives thereof, will naturally occur when such molecules are present in (or isolated from) aqueous solutions of various pHs. All salts of peptides and other macromolecules having the indicated biological activity are considered to be within the scope of the present invention. Examples include alkali, alkaline earth, and other metal salts of carboxylic acid residues, acid addition salts (e.g., HCl) of amino residues, and zwitterions formed by reactions between carboxylic acid and amino residues within the same molecule.

As will be readily appreciated in light of the present invention, homogeneous polymer conjugate compositions can be comprised of homo- or hetero-polymers. As discussed in the remainder of the application, when a heterobi- or heteromulti-polymer functionalized-polymer is attached to a target macromolecule not all of the polymer molecules attached at the single site will be identical with respect to chemical type or MW(av), nevertheless the composition can be homogeneous as defined herein. For example, step-wise assembly of polymers onto a linking structure ("L") (see co-pending United States Serial Numbers 08/057,594, 08/114,877, and 08/057,594, and co-pending International application PCT/IB94/00093, which are hereby incorporated by reference) allows the introduction of different polymers at each step to create a homogeneous composition of a hetero-polymer.

As noted above, parallel assembly of multi-polymer constructions by chemoselective ligation is the result of the complementary chemical reaction between, for example, a GXL group on the linking structure (L) and an AoA group on the functionalized polymer to form a homogeneous preparation of a multi-polymer construction having a defined macromolecular structure. Other embodiments of multi-polymer functionalized polymers have the reverse complementary structures, i.e. amino-oxy linking structure and aldehydic functionalized polymer, from those described above.

A additional polymer conjugate embodiment of the invention is a "dumbbell" construction wherein two target macromolecules, either the same or different, are each attached via oxime linkages to an intervening polymer, preferably through the termini of the polymer. A preferred embodiment comprises PEG or its derivatives as the intervening polymer.

Provided by the present invention are methods of polymer conjugate formation including the step of reacting a functionalized polymer and a functionalized target molecule to form an oxime linkage. An oximation reaction will occur site-specifically between the two reagents such that the polymer will be grafted to the target macromolecule by an oxime linkage. For example, an oximation reaction can occur between GXL-functional group introduced on a target macromolecule and its complementary AoA group introduced on a polymer at pH 4.6 to form the oxime-conjugate. Oximes form over a wide range of pH values and form rapidly at pH values less than about pH 5. The extent of oxime formation can be monitored by RP-HPLC and the reaction can be terminated by preparative RP-HPLC. The molecular weight of the resulting compound can be determined by gel electrophoresis or matrix-assisted laser desorption ionization mass spectrometry. Alternatively, an AoA-target macromolecule can be used, and an oximation reaction can occur between the AoA-group and a polymer having an aldehyde group. Alternatively, oximation is run at pH below 4.6. Lower pH can be advantageous for the solubility of some peptides. A pH of 2.1 is preferred for increasing the solubility of some peptides. In addition oximation occurs much faster at pH 2.1 than at pH 4.6. One skilled in the art can determine the pH versus solubility profile of a polymer and a target for oxime-conjugate formation and choose an appropriate

pH for a specific oximation reaction, taking into account pH stability of the molecules during the period of the oximation reaction. Oximation due to chemoselective ligation of the complementary chemical groups results rapidly and essentially quantitatively in the formation of a homogenous preparation of a oxime conjugate of defined structure when the AoA and GXL pair are used.

Oxime-forming complementary chemically reactive groups can be attached in either a protected or an unprotected form. Methods to attach an oxime-forming complementary chemically reactive group to a target macromolecule include attachment through a chemically reactive side chain group. For example an oxime-forming complementary chemically reactive group can be attached to a cysteine-containing target macromolecule via the S atom by alkylation or disulfide formation. Then upon oximation to a functionalized polymer having an aldehydic function the target macromolecule is attached via its Cys residue through a thioether link (or disulfide bond) and an oxime link to the polymer. Preferred alkylating compounds are alkyl halides having an attached AOA group. Specifically preferred are those having a BOC protected amino-oxy group preferably an AoA group. Preferred are Br-CH2-CO-NHCH2CH2NH-CO-CH2-O-NH-Boc, where the AoA group is protected and can be removed prior to an oximation step, and Br-CH2-CO-NHCH2CH2NH-CO-CH2-O-NH_2. Another alkylating reagent is Br-$CH_2CH_2CH_2$NH-$COCH_2$ONH-Boc. The bromoacetyl group is much more reactive for alkylation of the thiol group of, for example, Cys residues. Less preferred is the iodoacetyl group because it sometimes is too reactive and may be lost by photolysis. Other alkylating groups, in addition to the bromoacetyl group, include the maleoyl group. As taught herein, linkers for protein modification using this group are exemplified as AOA-Lys(maleoyl-beta-alanyl)-OH and maleoyl-beta-alanyl-$NHCH_2CH_2NH$-$COCH_2ONH_2$. Although the maleoyl group is useful for making macromolecular conjugates, it is known to have serious stability problems (hydrolytic opening of the ring) and so is less suitable for making homogeneous polyoximes. Furthermore, alkylation involving the maleoyl group gives a linker which is more rigid and bulky than the link formed by alkylation with the bromoacetyl group, and is thus more visible to the immune system. A preferred linker for in vivo application is one against which an immune response is not

directed. Examples of compounds for attachment of a oxime-forming complementary chemically reactive groups to the side chain of cysteine through a disulfide bond are those containing a 2-pyridyl-S-S- radical. Preferred examples are 2-pyridyl-S-S-CH2CH2NH-CO-CH2-O-NH-Boc and 2-pyridyl-S-S-CH2CH2NH-CO-CH2-O-NH_2. The resulting Cys-containing derivatives possess an aminooxyacetyl (or protected aminooxyacetyl) group attached through a disulfide bond. The modification disclosed herein is useful for connecting functionalized polymers to a polyaldehyde baseplate via a Cys side-chain through disulfide and oxime bonds. With this form of attachment, target macromolecules, e.g. peptides, can be liberated from the polymer by disulfide reduction, a process which is known to occur in the body.

The oxime-linked polymer-conjugates and the polyoxime based multi-polymer functionalized polymers of the invention have several novel characteristics. One novel characteristic is that the preparations are homogeneous. The oxime-linked compounds are stable in aqueous solution or semi-aqueous solution, and can be prepared at temperatures from -3°C to 50°C, but most advantageously at room temperature. The oxime-linked compounds of this invention have utilities related to the specific biological reactivity and specific chemical and physical reactivities of their individual component parts.

After the functionalized polymer of the invention (be it a mono-functional polymer, a bi-functional polymer, a bi-polymer functionalized polymer, or a multi-polymer functionalized polymer) is obtained, it is conjugated with a functionalized target macromolecule via an oximation reaction. An oxime linkage is formed from the complementary functional groups that had been introduced onto the polymer and target macromolecule. The grafting of the polymer to the target molecule occurs site-specifically as directed by the selected placement of the oxime-forming functional group on the target macromolecule.

In one embodiment of the invention, the method of conjugation is performed in the following manner. A functionalized polymer solution is combined with a functionalized target macromolecule solution at a final acidic pH. The oximation reaction is allowed to proceed to formation of the desired conjugate. As discussed above, the oximation reaction can occur over a wide range of pHs,

preferably acidic to about pH2, more preferably less than about pH 5, particularly when rapid formation is desired. More preferably, the pH is less than 4, most preferably about 3.6. The oximation reaction is compatible with lower pH values when it is necessary for maintaining target molecule stability or solubility. The reaction temperature is preferably room temperature, but can be adjusted to meet the specific needs for stability and solubility of the target macromolecule. The reaction time is between 10 minutes and 72 hours, more preferably between 3 hours and 48 hours, and most preferably between 6 hours and 24 hours. The functionalized polymer is in molar excess to the functionalized target macromolecule, preferably between about 3-fold to 20-fold molar excess, more preferably between about 4-fold to 15-fold, and most preferably between about 5-fold to 10-fold molar excess.

The functionalized polymer is preferably diluted in aqueous, preferably buffered, solution at acidic pH. Acetate, 0.1M, pH4.6 is a preferred solution. Optionally, a chaotropic agent is present in order to aid in accessibility of the functional group of the target macromolecule to the functionalized polymer. A preferred agent should be inert to the oximation reaction, inert to both the polymer and target molecule, and will not prevent the return of biological activity (if it was affected by the chaotropic agent) when removed from the final product. A preferred agent is guanidine hydrochloride ("GuHCl"), preferably at about 1 to 8 molar concentration in the final reaction, more preferably about 4 to 6 molar concentration. If the aminooxy derivative is not soluble in water, the conjugation reaction can be performed in the presence of an organic solvent. The solvent, and its concentration, should not irreversibly interfere with the biological activity of interest of the target macromolecule. Suitable solvents are well-known in the field. For example, for certain poorly soluble peptides, up to 50% acetonitrile can be used for coupling.

If desired, the polymer conjugate can be purified from the reaction mixture. There are many purification methods that are known to those of ordinary skill in the art such as size exclusion chromatography, hydrophobic interaction chromatography, ion exchange chromatography, preparative isoelectric focusing, etc. One particularly preferred method is to combine a size separation method with

charge separation method, for example, size exclusion chromatography followed by ion exchange, see U.S. Ser. No. 253,708, which is hereby incorporated by reference in its entirety. Preferably, the size separation method is size exclusion chromatography which discriminates between molecules based on their hydrodynamic radius. Hydrodynamic radius is defined as the effective molecular radius of a particle in an aqueous environment. Preferably, the charge separation method is ion exchange chromatography which discriminates between molecules based on differential affinity of charged ions or molecules in solution for inert immobile charged substances. The size exclusion chromatography method and the ion exchange chromatography method comprise contacting a mixed solution of polymer conjugates with either column in the appropriate buffers and under the appropriate conditions.

In another embodiment of the invention, the method of conjugation includes a "one-pot" synthesis. A functionalized polymer solution can be simultaneously mixed with both a periodate solution and a target macromolecule having an N-terminal residue of serine or threonine, which is highly susceptible to functionalization as described herein, under mild reaction conditions. Formation of the desired polymer conjugate, with the polymer site-specifically attached via an oxime linkage to the N-terminal amino acid of the target macromolecule will occur without the need for additional manipulation. The pH of the "one-pot" reaction is from about 2 to 9, preferably from about 3 to 7, more preferably about 4.5 to 7, most preferably about 6 to 7, with a preferred embodiment at about pH 6.5. Both the polymer and periodate are in molar excess to the target molecule. The periodate is preferably in the molar excess range of 2-fold to 5-fold, more preferably 2- to 3-fold. Reaction times of less than about 3 hours are achieved. If desired, the polymer conjugate is isolated as described herein. The reaction temperature is between preferably about 0°C to avoid freezing to below whatever temperature might denature the target macromolecule, generally below about 100°C to avoid denaturation, and in the case of most protein about 50° C. More preferably the reaction is at about 15 to 25°C, even more preferably about 20°C. The molar ratio of functionalized polymer to target is a value preferably in the range between about 3-fold to 50-fold, more preferably 4-fold to 30-fold, even more preferably about 5-

fold to 25-fold. The molar ratio of target molecule to periodate is preferably in the range of about 1-fold to about 6-fold, more preferably about 2-fold to 4-fold. In a specific embodiment, Il-8:$NaIO_4$:AoA was approximately 1:4:10.

To determine which fractions contain the desired conjugate, the fractions can be screened against various standards. Preferred screening methods include SDS-PAGE, isoelectric focusing, bioactivity, and pharmacokinetics.

Once it is known which fraction contains the desired conjugate, those fractions may be further purified.

For example, the polymer/protein conjugate mixture can be fractionated with the size exclusion chromatography column, the fractions collected, then run on an SDS-PAGE gel to determine which fractions contain the desired polymer/protein conjugate. Then, the fractions of interest may be further purified by contact with the ion exchange chromatography, the fractions collected, and analyzed by isoelectric focusing to determine which fractions have the desired polymer/protein conjugate. Before the polymer conjugate mixture is subjected to chromatography, it can be initially prepared by removing impurities. For example, salts and chaotropic agents can be removed with preparatory columns, or can be dialyzed against appropriate buffers.

Once the polymer conjugate is purified it can be tested for bioactivity using methods known in the art.

According to a preferred embodiment of the present invention, protein and other organic target macromolecules may be chemically modified by conjugation to water-soluble organic polymers such as polyethylene glycol (PEG). The production of such protein conjugates is of interest because of the desirable properties conferred by the attachment of the water-soluble polymers. These desirable properties include increased solubility in aqueous solutions, increased stability during storage, reduced immunogenicity, increased resistance to enzymatic degradation, compatibility with a wider variety of drug administration systems, and increased in vivo half-life. These properties that are brought about by the derivatization of polypeptides with PEG or other water-soluble polymers are especially of interest when the polypeptide is to be used as a therapeutic agent

injected into the body or when the polypeptide is to be used in assays, usually immunoassays, for the detection and/or quantification of a compound of interest.

Naturally, the utility of the functionalized polymers of the invention extends to preparation of polymer-conjugates of low molecular weight peptides and other materials that contain or are modified to contain a functional group complementary to that on the polymer.

Non-medical uses of functionalized polymers, particularly PEGylated proteins include the preparation of polypeptides for assays, e.g. immunoassays,

The functionalized polymers can be attached to a solid phase, such as the surface of a silicon chip, a tissue culture plate, cell or membrane, or a synthetic or natural resin. One can chemoselectively ligate a functionalized polymer to a solid phase through the use of complementary functional groups introduced to the solid phase.

As demonstrated herein, the functionalized polymers are useful to monitor the appearance (or disappearance) of a functional group on a target macromolecule or solid phase.

After the polymer conjugate is produced and purified it may be incorporated into a pharmaceutical composition when target macromolecules believed to be therapeutically effective for human and veterinary uses, such as cancer therapy and the treatment of infectious diseases have been used.

A therapeutic agent is any molecule, which, when administered to an animal, prevents or alleviates a disease or arrests or alleviates a disease state in the animal. Therapeutic agents may include, but are not limited to, antitumor antibiotics, antiviral proteins, radioisotopes, pharmaceuticals or a toxin.

The polymer conjugate can be formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium. A "pharmaceutically acceptable carrier" means any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution; water; or emulsion, such as an oil/water emulsion; potentially including various types of wetting agents. The polymer conjugate can be formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium, preferably at a pH ranging from 3 to 8, more preferably

ranging from 6 to 8. When used for in vivo therapy, the sterile polymer conjugate composition will comprise protein dissolved in an aqueous buffer having an acceptable pH upon reconstitution. The polymer conjugate can be formulated with a number of excipients such as amino acids, polymers, polyols, sugar, buffers, preservatives, other proteins, etc. Specific examples include: octylphenoxy polyethoxy ethanol compounds; polyethylene glycol monostearate compounds; polyoxyethylene sorbitan fatty acid esters; sucrose; fructose; dextrose; maltose; glucose; dextran; mannitol; sorbitol; inositol; galactitol; xylitol; lactose; trehalose; bovine or human serum albumin; citrate; acetate; Ringer's and Hank's solutions; saline; phosphate; cysteine; arginine; carnitine; alanine; glycine; lysine; valine; leucine; polyvinylpyrrolidone; polyethylene glycol; etc. Preferably this formulation is stable for at least 6 months at 4°C.

As a composition, it is parenterally administered to the subject by methods known in the art. Administered means providing the subject with an effective amount of the compound or pharmaceutical composition. Methods of administration to an animal are well known to those of ordinary skill in the art and include, but are not limited to, oral, intravenous, transdermal, and parenteral administration. Administration may be effected continuously or intermittently throughout the course of other treatments. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the compound or composition for treatment, the purpose of therapy and the animal or patient being treated. This composition may contain other compounds that increase the effectiveness or promote the desirable qualities of the particular target macromolecule portion. The composition must be safe for administration via the route that is chosen, sterile and effective. To maintain the sterility and to increase the stability of a polymer conjugate, the composition can be lyophilized and reconstituted prior to use.

Preferably, the formulation is suitable for parenteral administration to humans or animals in therapeutically effective amounts. These amounts may be determined by the in vivo efficacy data obtained after preclinical testing for: animal models of the disease state of interest or in vitro assays generally accepted as correlating with in vivo efficacy.

It is also at interest to supply the water-soluble functionalized polymer reagents of the invention in the form of a kit, so as to provide for the convenient and reproducible derivatization of target macromolecules of interest. Kits of interest may contain solutions comprising the water-soluble functionalized polymer reagent of the invention, buffers, reaction indicator compounds, instruction, protein concentration measurement reagents, e.g., for Bradford assays, and the like. Reagent solutions will preferably be supplied in premeasured amounts.

Kits can contain reagents for site-specifically introducing a complementary functional group onto a target macromolecule of interest, preferably under mild conditions. Such reagents can include a functionalized polymer of the invention that allows the functionalization reaction to be monitored (as by formation of a polymer conjugate), peptidases for N-terminal cleavage, proteases for C-terminal site-specific conjugation, linker groups containing protected amino-oxy groups, periodate, molecular weight markers (as in the form of polymer conjugates of known molecular weight) and optionally any buffers or solvents for carrying out functionalization of a target macromolecule.

Kits can contain a series of individual solutions (or powdered form) polymer-conjugates containing polymers of known composition, molecular weight and configuration (whether mono-polymer, bi-polymer or multi-polymer) attached to target macromolecules of known molecular weight and composition that can be used as standards, for example to estimate completion and/or yield of conjugation reactions or to provide molecular weight standards.

Polymer-conjugate embodiments of the invention can be used in improved kits for diagnostic purposes or as improved reagents for assays, for example, in binding assays such as immunoassays. For example, polymer conjugate compositions bearing antigen peptides provide increased detection sensitivity in solid-phase immunoassays. The larger, bi-valent or multivalent polymer conjugates can more readily adhere to surfaces such as the multiwell plates used in immunoassays. Polymer conjugates, particularly multi-polymer-containing polymer conjugates, find use in in vitro assays that use a signal amplification step for detection of an analyte, as for example in a branched DNA ("bDNA") based assay. Amplification is achieved by the attachment of multiple polymers (rather than a

single polymer) to a single analyte molecule, wherein each polymer contributes to a detectable signal in a subsequent assay step. Targeting of the polymer conjugate to the analyte is readily provided, including for example by use of a linking L group that comprises an analyte binding group or by use of a hetero-polymer construct of the invention wherein at least one polymer on the construct provides analyte binding. Preferred are multi-polymer-containing polymer conjugates of the invention, particularly those in which the polymer comprises a specific nucleotide sequence as a repeating unit that is subsequently detected by hybridization to a labeled second oligonucleotide, wherein the label can be radioactive, fluorescent, enzyme-linked, or the like as is known in the art. Alternatively, the functionalized polymer can itself be derivatized to contain a detectable marker enzyme-link, or other reporter group.

General methods and principles for macromolecule purification, particularly protein purification, can be found, for example, in "Protein Purification: Principles and Practice" (1987) by Scopes, 2nd ed., Springer-Verlag, New York, NY, which is incorporated herein by reference.

Also provided herein are methods of systematically modifying the Stokes radius of an organic target macromolecule, including the steps of (a) obtaining a site-specifically-functionalized target macromolecule comprising a first oxime-forming group, (b) obtaining a series of functionalized organic polymers of the invention that differ from each other in the series in topology but not molecular weight(average) and that include a second oxime-forming group complementary reactive to the first oxime-forming group on the target macromolecule, and then (c) conjugating the functionalized target macromolecule separately with each functionalized polymer via a chemoselective, site-specific oximation, preferably under mild conditions as taught herein, to obtain a series of conjugated polymers. The steps (a) and (b) are performed in any order. If desired one can identify a change in Stokes radius for each conjugated polymer in the series, preferably by size separation methods. The method can further include correlating the change in Stokes radius with a change in a biological or physical property of interest of a target macromolecule. Alternatively, the method includes identifying a change in a biological or physical property of interest, e.g. pharmacokinetic behavior,

therapeutic efficacy, use in an in vitro assay system, behavior as a molecular weight standard, of the target macromolecule of interest. Preferably, the second oxime-forming group is -O-NH_2.

The in vivo blood clearance behavior of a macromolecule must in part be dependent on its Stokes radius. The elution volume of a macromolecule from a gel filtration column, relates theoretically to the Stokes radius, not to its molecular weight.

Mobility of a macromolecule when subject to gel electrophoresis, wherein a three-dimensional network of filaments form pores of various sizes for filtering of migrating molecules, can reflect changes in the Stokes radius of a macromolecule. Typically, polymers varying in molecular weight will migrate as a linear function of the log of molecular weight (and where the charge to mass ratio for each polymer is approximately the same).

In one embodiment of the present invention, functionalized polymers of varying topology, but of similar molecular weight, are provided that allow one to impart a desired change in the Stokes radius of a target macromolecule, more preferably create a family of molecules by systematic introduction of a series of changes in Stokes radius.

A series of conjugated target macromolecules can be rapidly prepared in which either a mono-polymer, a bi-polymer or a multi-polymer-containing functionalized polymer construct is site-specifically, chemoselectively attached at a unique site, wherein each attached functionalized polymer construct adds the same molecular weight and polymer composition but in a differing topology as the others to the target. However, as discovered herein, because of their resulting varying polymer configurations, the mobility of each polymer conjugate is surprisingly different. By systematically modifying the Stokes radius of a target macromolecule as taught herein, one can now more readily create (and "fine-tune") and identify those conjugated molecules having enhanced or desired behavior.

Advantages of coupling water-soluble polymers, especially polyethylene glycol, to proteins have been well documented and include the following: increased solubility of the conjugated protein as compared with the native protein at physiological pH when native protein is insoluble or only partially soluble at

physiological pH, a decrease in the immune response generated by the native protein, an increased pharmacokinetic profile, an increased shelf-life, and an increased biological half-life.

By systematically modifying the Stokes radius of a target macromolecule, particularly a pharmaceutically important protein as taught herein, one can in turn systematically impart changes in the pharmacokinetic behavior of the molecule, which will enable one to more readily create and identify those conjugated molecules having enhanced or desired behavior or therapeutic efficiency. Thus, functionalized polymers having different polymer topologies while keeping the molecular weight (average) constant are provided. For example, a family of functionalized PEG polymers of similarly weighted PEGs with differing topologies are provided such that the Stokes radius, and thus pharmacokinetic behavior of a biologically important target molecule, can be changed in predictable and reproducible ways.

Kits are provided that contain a series of functionalized polymers for attachment to a target of interest, wherein the series comprises polymer constructs of similar molecular weight but varying in the number of polymers present in the construct. For example, a series can comprise constructs of a 40 kD MW(av) polymer, and multi-polymer-containing construct of two 20 kD MW(av) polymers, four 10 kD MW(av) polymers, and five 8 kD MW(av) polymers.

The present process is advantageous because the attachment of polymer(s) to a target is predictable and selective.

A further advantage of the subject invention is that macromolecules, e.g. polypeptides, modified by the reagent compounds retain a greater degree of their biological activity than when the same target is modified to the same degree by joining water-soluble polymers by employing non-oxime and non-site-specific chemistries. Thus, the subject invention provides for modified targets that possess the advantages associated with the conjugation of water-soluble polymers while minimizing the loss of biological activity associated with the modification. Consequently, the targets that are more highly derivatized by the use of multi-polymer functionalized polymers, and thus possessing the advantages associated with the higher degree of derivatization, can be produced that have the same level of

biological activity as polypeptides derivatized by water-soluble polymers to a lesser extent.

An additional advantage of the present invention over other methods of coupling water-soluble polymers to proteins, e.g., the use of active carbamates, such active esters can react with nucleophiles other than primary amines such as hydroxyl groups, phenolic groups, and sulfhydryl groups on a protein, and the use of imidates that selectively react with the primary amino group of lysine residues, is that cross-reactivity is avoided because of the highly selective nature of the particular chemistry of the invention.

An additional advantage is that the site-specificity can be precisely located on the target macromolecule by use of the site-specific functionalization methods and linkers as provided by and as taught herein.

As demonstrated herein, the complementary reactive groups that interact to form an oxime linkage between polymer and target are highly specific. The oximation reactions taught herein provided complete or essentially quantitative yield of the reaction product. Such complex molecule formation occurs under very mild conditions. Rapidity is particularly surprising under dilute conditions which are often useful to minimize inter-molecular aggregation or reactions. The oximation reaction can occur unattended, such that self-assembly of the polymer-conjugate takes place. Polymer conjugates are easily purified by virtue of the essentially quantitative yield and because trace intermediates and the final product typically differ substantially (*i.e.*, by the presence or absence of at least one polymer unit) so that methods for their separation are readily chosen and applied. Oxime linkages have superior hydrolytic stability over a range of physiological conditions compared to hydrazones or the like. Oxime linkages are not commonly subject to enzymatic hydrolysis. Thus polymer conjugates have the advantage of being particularly suited to applications where integrity and stability of a complex is desirable. As demonstrated in the examples, the oximation reaction is very mild and thus is suitably advantageous for preparing biological macromolecules retaining biological activity or function. The oxime chemistry dispenses with the need to have reversible chemical protection of subunits. A great flexibility is provided herein for site-specific modification of both polymers and targets to create reactive groups

capable of forming oxime linkages. Because of this flexibility and the absence of the need for reversible protection, the design of polymers and targets extends to both artificial and natural molecules and their derivatives. Polymer-conjugates can be designed to improve solubility of peptides as well as present peptides to receptors or antibodies or the immune system of an animal in multi-valent and/or constrained forms. Polymer-conjugates formed from synthetic or recombinant polymers and target macromolecules have the additional advantage of being virus free.

Accordingly, preferably the functionalized polymers of the invention and the methods provided herein allow a polymer to be conjugated to polypeptide and protein derivative by a condensation reaction between an aldehyde and an amino-oxy compound. Most preferably the complementary reaction is between a aldehyde or ketone and an amino-oxy-acetyl. In all cases an oxime bond is formed.

The present invention provides further *in vitro* use of polymers, such as PEG. A functionalized polymer can be used to "tag" a target molecule and thus enable the molecule's subsequent detection and or quantitation in a number of ways. Most simply, the attached polymer allows one to perform a simple size separation that will separate the polymer tagged-target molecule from other molecules in a mixture. For example, one can readily follow the modification of a target molecule for the production of desired functional sites as measured by the ability to react with the reactive functional group on the functionalized polymer, which is in turn detected as the appearance of the polymer-tagged target. It is now readily apparent that different physicochemical properties of organic polymers can be taken advantage of in this way simply by changing the polymer. For example, a slightly hydrophobic polymer would allow separation based on hydrophobicity, or one can use a polymer binding column that then selects for or against the polymer-conjugate as desired. In addition the polymer can chosen, or modified, so that it can be directly detected. This imparts the advantage that the polymer may contain multiple detectable sites (or repeating units), such that each site present in the polymer binds or is recognized by a detection system, thus resulting in the amplification of detection signal. Branched DNA ("bDNA") containing reagents exemplify the case wherein each polymer unit, in this case a specific nucleotide sequence or repeating sequence, is detected by specifically binding a second measurable reagent (Urdea

(1994) Bio/Technology 12: 926-928). For example, in the copper catalyzed transamination ration presented herein, the appearance of the reactive keto group at the N-terminus of a target protein could not be easily assessed. Disappearance of the N-terminal amino group could not be assessed by either mass spectrometry since only the copper adduct could be identified, or by cellulose acetate electrophoresis. However, the appearance of the reactive keto group was easily assessed by its ability to react with a functionalized PEG derivative of the invention as detected, for example, by simple size separation in an SDS-PAGE system.

EXAMPLES

EXAMPLE 1. Functionalization of Methoxypolyethylene Glycol: Synthesis of MPEG-NH-CO-CH_2-O-NH_2 ("AoA-NH-PEG").

$MPEG_{5kD}$ (5g, 1mmol; M_r(av) 5 kD) was dissolved in toluene (30ml) and dried by azeotropic distillation. Dry pyridine was added and thionyl chloride (4mmol) was added dropwise during 10min under reflux. The mixture was heated for 4hr, cooled to room temperature, filtered from pyridine hydrochloride and the toluene was evaporated in vacuo. The residue was dissolved in CH_2Cl_2, dried over K_2CO_3 and filtered. The filtrate was treated with alumine oxide (40g) and precipitated by cold ether, the polymer was recrystallized from toluene/hexane; yield 4.6g (92%).

To a solution of $MPEG_{5KD}$-Cl (4g, 0.8mmol) in DMF (20ml), sodium azide (8mmol) was added and the mixture was stirred at 120° for 2 hr. The solution was cooled, filtered and the DMF evaporated in vacuo. The residue was taken up in toluene, filtered and precipitated with hexane. The product was recrystallized twice from toluene /hexane ; yield 3.6g (90%).

$MPEG_{5kD}$-N_3 (3.5g) was dissolved in absolute ethanol (100ml), 10% Pd/C (0.3g) added and the mixture was hydrogenated in a low pressure hydrogenation apparatus over night. The catalyst was filtered and ethanol evaporated in vacuo. The polymer was recrystallized from toluene/hexane; yield 3g (86%).

$MPEG_{5kD}$-NH_2 (1g, 0.2mmol) and Boc-NH-O-CH_2-COOSu (0.5mmol) were dissolved in DMSO (4ml), the apparent pH adjusted to 8-9 with N-methylmorpholine and the mixture stirred over night. MPEG-NH_2 acylation was

controlled by the quantitative ninhydrin procedure (Sarin et al. 1981 Anal. Biochem. 117:147-157) on a 10ul aliquot of the reaction mixture. The modified MPEG was then recovered by dilution with 5 volumes of water, followed by dialysis against water, dialysis and lyophilization. The Boc group was removed by dissolving the product in 10ml of TFA for 1hr at room temperature. TFA was removed under vacuo, the material was taken up in water, extensively dialyzed against water and finally lyophilized; yield 0.93g (93%).

The same procedure was used for the synthesis of AoA-NH-$PEG_{10\ kD}$ and AoA-NH-$PEG_{20\ kD}$ from corresponding PEG-NH_2.

EXAMPLE 2. Functionalization of Methoxypolyethylene Glycol: Synthesis of NH_2-O-CH_2CONH-PEG-NHCO-CH_2-O-NH_2 ("AoA-NH-PEG-NH-AoA").

Boc-NH-O-CH_2-COOSu (86mg, 0.3mmol) was dissolved in 1ml dry DMSO, added to a solution of 1g (50umol) of NH_2-PEG_{20kD}-NH_2 in 4ml of the same solvent and the apparent pH adjusted to 8-9 with N-methylmorpholine. The mixture was stirred at room temperature overnight and acylation controlled by the standard ninhydrin analysis. The solution was then diluted with 5 volumes of distilled water, extensively dialyzed against water and finally lyophilized. The Boc group was cleaved and the material further worked up as described in Example 1; yield 0.9g (90%). Conjugation with this polymer leads to dimer, e.g. "dumbbell" formation.

EXAMPLE 3. Functionalization of Methoxypolyethylene Glycol: Synthesis of Multivalent Linker $(PEG)_2$Lys-NH-$(CH_2)_2$-NH-CO-CH_2-O-NH_2 ("$(PEG)_2$Lys-AoA").

To 2.0g (3.9mmol) of Z-Lys(Z)-OSu was added 4ml ethylene diamine (60mmol) and the mixture stirred for 2hr at room temperature, where Z is benzyloxy-carbonyl. The coupling reaction was quantitative as controlled by analytical HPLC on a C8 column using a linear gradient of 0-100% B over 50min (t_R= 41.5min instead of 48.5 for Z-Lys(Z)-OSu). The coupling product was purified by flash chromatography on a silica column equilibrated in $CHCl_3$/MeOH (9/1,v:v) and dried by rotary evaporation to yield 1.4 g material. The product had

the expected molecular weight, as determined by ESIMS (Calcd M+H, m/z 457.2 ; found m/z 458.0).

Z-Lys(Z)-NH-$(CH_2)_2$-NHCO-CH_2-O-NH-Boc was synthesized by adding 1.1 g Boc-NH-O-CH_2-COOSu (3.8mmol) to 0.9g of Z-Lys(Z)-NH-$(CH_2)_2$-NH_2 (1.9mmol) dissolved in 3ml of dry DMSO and the apparent pH adjusted to 8-9 with N-methylmorpholine. The solution was stirred during 5h at room temperature, diluted with 10 volumes of 0.1% TFA and the product purified on a preparative HPLC column 250x 25mm i.d. (Nucleosil 300A, 7um C8, Macherey Nagel, Oensingen, Switzerland) using a linear gradient from 40 to 80%B over 30min with a flow rate of 3ml/min. After lyophilization, the product (weight, 1.1g; yield 90%) was characterized by ESIMS (calcd M+H, m/z 630.2, found m/z 630.9).

The Z group was cleaved by catalytic hydrogenation. For this purpose, the material was dissolved in absolute ethanol (50ml), acidified with 100ul CH_3COOH, 10% Pd/C (0.1g) added and the mixture hydrogenated in a hydrogenation apparatus overnight. The catalyst was filtered and the solvent removed by rotary evaporation. The product was purified on the same column than previously used with a linear gradient from 0 to 50%B over 25min. After lyophilization (weight, 600mg; yield, 95%) the product was characterized by ESMS: calcd M+H, m/z 360.2; found m/z 361.8).

Lys-NH-$(CH_2)_2$-NHCO-CH_2-O-NH-Boc was acylated with PEG_{5kD}-COOH, and PEG_{10kD}-COOH, and PEG 20KD-C00H.

In a typical experiment, 14mg of Lys-NH-$(CH_2)_2$-NHCO-CH_2-O-NH-Boc.2 HCL (33 umol) was dissolved in 2ml of dry DMSO, PEG_{5kD}-COOH (0.4g, 80umol), hydroxy-benzotriazole 80umol) and DCC (21mg, 100umol) were added the apparent pH adjusted to 8-9 with N-methyl-morpholine and the mixture stirred overnight. The standard ninhydrin test showed that acylation was quantitative. The solution was then diluted with 5 volumes of water, dialyzed against distilled water, filtered and applied on a DEAE Sephadex A25 column equilibrated in water. The acylated linker was eluted in the first fraction with distilled water and was lyophilized.

The Boc group was then cleaved by dissolving the product in 10ml of TFA for 1hr at room temperature. TFA was removed under vacuo, the material was

taken up in water, extensively dialyzed against water and finally lyophilized (yield, 300mg, 90%), to get the following derivative: PEG_{5kD}-Lys(PEG_{5kD})-NH-$(CH_2)_2$-NHCO-CH_2-O-NH_2.

A comparable linker was obtained by acylation of the lysine derivative with PEG_{10kD}-COOH, and PEG_{20KD}-COOH.

EXAMPLE 4. Functionalization of Methoxypolyethylene Glycol: Synthesis of Multivalent Linker AoA-(Lys(PEG_{5kD}))$_5$-OH.

Z-(Lys(Boc))$_5$-OH was used as starting material. The peptide, once the Boc-group was removed, resolubilized in 10mM HCl, relyophilized and then was completely acylated with 2-fold excess of PEG_{5kD}-COOH in DMSO in the presence of HOBt and DCC. The reaction mixture was diluted with distilled water, dialyzed and the soluble fraction further purified by ion exchange chromatography on a DEAE-A25 an CM-C25 Sephadex column, and lyophilized. The Z-group was cleaved by a 2h HBr treatment and the product was then acylated with Boc-AoA-OSu. Finally, the AoA function of the linker was deprotected by TFA treatment. The extent of functionalization could be evaluated by determination of reactive aminooxy groups with TNBS at 495nm, and was found to be 80%.

EXAMPLE 5. Functionalization of Dextran: Synthesis of Dextran -O-CH(CHOH-CH_2OH)-(CHOH)$_2$-CH_2-NH-$(CH_2)_2$-NH-CO-CH_2-O-NH_2.

A unique reactive group was introduced in dextran ($Mr_{(av)}$9.3 kD and 39kD) by modification of the reducing sugar by reductive amination.

$Dextran_{39kD}$ (1g, 25umol) was dissolved in anhydrous DMSO (4.5ml) with gentle heating (45°C) and allowed to return to 25°C. Ethylene diamine (500ul, 7.5mmol) and crushed 4-A molecular sieves (200mg) were added. The flask was flushed with N_2 and then sealed and incubated for 24h at 37°C. $NaBH_4$ (80mg, 2.1mmol) was added, the flask flushed with N_2 and the solution further incubated for 24h at 37°C. The viscous solution was then diluted with 5 volumes of water, the pH adjusted to 5.0 by the addition of glacial acetic acid, extensively dialyzed against distilled water and finally lyophilized to afford 800mg (80% recovery on the based on starting dextran). The yield of functionalization was estimated from the

absorbance of the 2,4,6-trinitrobenzene sulfonic acid derivative at 420nm (Fields, R., 1972, Methods Enzymol., 25B, 464-468). A comparable extent of end-group modification was obtained for $dextran_{9kD}$ and $dextran_{39kD}$, (0.9 to 1.0 mole NH_2 per mole polymer) and was in agreement with that obtained for mono functionalized commercial $MPEG_{10kD}$-NH_2 and $MPEG_{20kD}$-NH_2.

NH_2-derivatized $dextran_{39kD}$ ((800mg, 20umol) was dissolved in DMSO (5ml), Boc-NH-O-CH_2-COOSu (19mg, 60umol) dissolved in the same solvent (1ml) added, the apparent pH adjusted to 8-9 with N-methylmorpholine and the solution stirred overnight at room temperature. The solution was diluted with 5 volumes of water, extensively dialyzed against distilled water and lyophilized. The end-modified dextran was quantitatively acylated as controlled by the TNBS test. The material was dissolved in 20ml TFA during 1h for deprotection, TFA removed under vacuo, diluted with water, extensively dialyzed and freeze-dried.

The polymers functionalized with a single aminooxy group to their already introduced terminal amino group are able to react, preferably under acidic conditions, most preferably pH 3.0-5.0, with a carbonyl function, for example one located at or site-specifically introduced at the N-terminus of a protein by, for example, periodate oxidation or transamination (as dictated by the particular N-terminal sequence of the protein), to form a stable oxime bond. Site-specific placement of a carbonyl group at the N-terminus of a protein is demonstrated in the Examples below. Il-8, G-CSF and Il-1ra were used to illustrate this novel approach for site specific conjugation of aminooxy functionalized synthetic polymers.

EXAMPLE 6. Site-Specific Modification of NH_2-Terminal Residue of a Protein: Modification and Conjugation of IL-8.

The N-terminal serine residue of the short form of IL-8 (72 residues; 2-5 mg/ml) was oxidized with a 10 fold excess of sodium periodate in a 1% NH_4HCO_3 buffer at pH 8.3, in the presence of 50 molar excess of methionine during 10min at room temperature. Though IL-8 does not contain any methionine these standard conditions have been developed to eliminate all risk of methionine oxidation during periodate treatment (Gaertner et al. 1992 Peptides 1992, Schneider and Eberle, eds, pp239-240, ESCOM, Leiden, The Netherlands). The reaction was

stopped by the addition of 2000 excess ethylene glycol over periodate and further incubated for 15min at room temperature, and the protein was finally dialyzed against a 0.1M AcONa buffer, pH 4.6. Dialysis tubing was previously boiled in 1% sodium bicarbonate during 30min. Oxidation was confirmed by ESI-MS (calcd., m/z 8351.3; found m/z 8351.3±1.4). The oxidized protein was concentrated up to 3-4mg/ml and was used in that form for the site specific attachment of the functionalized polymers to the N-terminus of the protein backbone.

Conjugation with the polymer. A 10-fold molar excess of a 10mM aqueous solution of polymer was added to the oxidized protein, the pH adjusted to 3.6 with glacial acetic acid and incubated for 20 h at room temperature. As shown in Figure 1, SDS polyacrylamide gel electrophoresis clearly demonstrates that a homogenous conjugation product corresponding to the attachment of a single polymer chain is formed. The molecular weight of the conjugate is directly related to the size of the polymer used in the coupling reaction.

The conjugation product was purified by ion exchange chromatography on a Pharmacia MonoS column equilibrated in 25mM AcONa buffer, pH 4.7 using a linear gradient from 0 to 2M NaCl over 20min followed by reverse phase HPLC on an analytical C8 column using a linear gradient from 30 to 65% B over 35 min (Figure 2).

In vitro activity of conjugates. The biological activity of Il-8 conjugates was determined by a human neutrophil chemotaxis assay using a Micro-Boyden chamber as already described (Ribaudo and Kreutzer (1985) in Practical Methods and Clinical Immunology (Yoshida T. ed.). pp. 116-125, Churchill Livingston, Edinburgh, U.K.).

While Il-8 showed an EC_{50} of 2 nM (concentration corresponding to 50% of the maximal chemattractant activity), all investigated PEG-derivatives (PEG_{5kD}-, PEG_{10kD}-, PEG_{20kD}-, and $(PEG_{20kD})_2$Lys-Il-8 and Il-8-PEG_{20kD}-Il-8) were shown to have an activity of the same order of magnitude with an EC_{50} ranging between 3 and 10 nM.

Pharmacokinetic data. Il-8 and its derivatives were iodinated under conditions, which have already been shown to retain full biological activity as

determined by neutrophil chemotaxis (Grob et al. (1990) J. Biol. Chem. 265:8311-8316).

Briefly, approximately 0.5mCi of $Na^{125}I$ was mixed in a lml polypropylene tube with 50 μl of 0.1M sodium phosphate, Ph 7.4 containing 50 μg of Il-8. The iodination was initiated by addition of 15 μl of 2mM chloramine T.

Following a 90-s incubation at 20°C, the iodination reaction was terminated by addition of 15 μl of 2mM sodium bisulfite and 10 μl of 50 (v/v) potassium iodide. The radiodinated protein was separated from free ^{125}I on a GF5 column (Pierce) equilibrated in PBS and previously washed with the same buffer containing 0.1% bovine serum albumin. The specific radioactivity obtained was in the range of 5-10 mCi/mg protein.

^{125}I-labelled Il-8 or its derivative was injected as a bolus in the tail vein of female Wistar rats (10 μg/kg) and samples of blood collected as described in example 8.

The curves of relative blood levels for Il-8, PEG_{20Kd}-Il-8, IL-8-PEG_{20Kd}-Il-8 and $(PEG_{20Kd})_2$Lys-Il-8 given intravenously to the rats are shown in Fig. 7. Conjugation of a single 20kD PEG chain has almost no influence on the area under the pharmacokinetic curve. Most importantly, the dumbbell and the $(PEG_{20kD})_2$Lys-derivative considerably increase this area.

A manual fit to these curves gives apparent first $T_{1/2}$ values of approximately 10 min for Il-8, 17 min. for PEG_{20kD}-Il-8, 30 min for Il-8 PEG_{20kD}-Il-8 and 60 min. for $(PEG_{20kD})_2$Lys-Il-8.

No detectable differences were observed in the apparent second $T_{1/2}$ among all protein derivatives, but while less than 1% of the initially observed radioactivity remained in circulation after 24 h for Il-8, 6% of initial amount were still present in case of (PEG_{20Kd})2Lys-Il-8.

EXAMPLE 7. Site-Specific Modification and Conjugation of NH_2-Terminal Residue of a Protein: Modification and Conjugation of rh-G-CSF.

Application of the same methodology to the modification of G-CSF involves the removal of N-terminal methionine, to reveal the threonine which will be, as well as serine in case of Il-8, the target for periodate oxidation.

Enzymatic cleavage of N-terminal methionine. The methionine residue was specifically removed by enzymatic digestion with kidney microsomal aminopeptidase (EC 3.4.11.2). G-CSF was concentrated to 5mg/mL in a Tris 50mM, 0.3% sodium lauryl sarcosinate, pH 8.0 buffer and then incubated with aminopeptidase (enzyme/substrate ratio, 1/20) for 20h at 37°C, in the presence of 10mM $MgCl_2$, 2.5mM PMSF and benzamidine, and 2mg/mL aprotinine. All these inhibitors were added to prevent any cleavage of the polypeptidic chain by any contaminating proteolytic enzyme present in the commercial aminopeptidase preparation. Aminopeptidase was then inactivated by adding EDTA at a 10mM concentration and the solution dialyzed at 4°C versus Tris 20mM, pH 8.0 and against water and the material finally purified by reversed phase HPLC on a 250x10mm i.d. Nucleosil C8 column, using a flow rate of 3mL/min and a linear gradient of 50-80% solvent B over 15min. The modified protein was characterized by ESI-MS. The ESI mass spectrum exhibited two series of multi-charged ions, the major one corresponding to *des*-Met^1-rhG-CSF (calcd., m/z 18820.8; found, m/z 18821.7±2.1) and the minor one to Met^1-rh-G-CSF (calcd., m/z 18951.9, found m/z 18956.1±5.5).

Oxidation of *des*-Met^1-G-CSF. The lyophilized material was dissolved in a 0.1M sodium phosphate buffer, pH 6.5 at 5mg/ml in the presence of 6M guanidine chloride, and oxidation performed with a 5-fold excess of periodate over the polypeptide. After 10min of incubation at room temperature in the dark, unreacted periodate was destroyed with a 1000-fold excess of ethylene glycol. Oxidation was confirmed by ESI-MS (calcd., m/z 18775.3; found, m/z 18777.9±2.7).

Conjugation of the polymer. A 5-fold excess of AoA-NH-PEG was added to the reaction mixture, diluted with 5 volumes of 0.1M AcONa buffer pH4.6 containing 6M guanidine chloride and concentrated to one volume on a Centricon microconcentrator. Dilution and concentration was repeated a second time. The pH was then adjusted to 3.6 and a second 5-fold excess of AoA-NH-PEG added, and the solution incubated 24h at room temperature. The solution was dialyzed against a 20mM sodium phosphate buffer pH 7.0 to discard the guanidine chloride and the conjugation product isolated by HPLC on a C8 column. As shown in Figure 3,

both PEG_{5kD} and PEG_{20kD}-G-CSF could be isolated by reverse phase HPLC, but in the case of PEG_{5kD}-G-CSF, this step was preceded by hydrophobic interaction chromatography on a polypropyl aspartamide column, using a linear gradient from 20mM sodium phosphate, 1,2M Na_2SO_4, pH 6.3 to 50 mM sodium phosphate, 5% (v.v) isopropanol, pH 7.4 over 15 min. followed by a 25 min linear gradient to 25% of a third buffer containing 25 mM sodium phosphate, 50% MeOH pH 7.1, with a flow rate of 0.6ml/min.

EXAMPLE 8. Site-Specific Modification and Conjugation of NH_2-Terminal Residue of a Protein: Modification by Transamination and Conjugation of IL-1-ra.

Transamination. Since the N-terminal sequence of IL-1ra is Met-Arg-Pro-Ser-Gly-, copper-catalyzed transamination was used for activation of the N-terminus of the protein, followed by conjugation of functionalized PEG of different molecular weights. The concentrated protein solution (40mg/ml) was diluted 10 times in a 2.5M sodium acetate buffer, pH5.5; $CuSO_4$ and glyoxylic acid were added to a final concentration of 2mM and 0.1M, respectively. After 1h incubation at room temperature, the reaction was stopped by the addition of solid EDTA. The mixture was then extensively dialyzed against a 0.1M NaOAc buffer, pH4.6, and the soluble fraction used for the conjugation reaction.

Conjugation of the polymer. Appearance of the reactive keto group was characterized by its ability to react with AoA-NH-PEG. A 10-fold excess of polymer was added to the transaminated protein, the pH adjusted to 3.6 with acetic acid, and the reaction mixture incubated 48h at room temperature. PEG_{5kD}-Il-1ra, PEG_{10kD}-Il-1ra and PEG_{20kD}-Il-1ra were thus constructed as confirmed in Figure 4.

The protein conjugates were purified by hydrophobic interaction chromatography using a poly-propylapartamide column (200x4mm, 5um, 1000A, PolyLC Inc., Columbia, MA), followed by a gel filtration on a BioSep-SecS-2000 Phenomenex column (600x7.8mm) and ion exchange chromatography on a Mono Q column.

In vitro activity of conjugates. Il-1ra inhibits, in a dose-responsive fashion, the effects of Il-1β on the induction of Prostaglandin$_2$ (PG_2) production by dermal or synovial fibroblasts (Arend et al. (1990) J. Clin. Invest. 85:1694-1699).

This bioassay allowed the evaluation of the biological activity of modified Il-1ra, by using a 1000-fold excess concentration of Il-1ra or its conjugate over Il-1β to study the inhibition of the Il-1β induced biological response. Table 1 presents the results of one such initial assay. The PEG_{20kD}-des(MRP)Il-1ra form listed in Table 1 is that derived by enzymatic cleavage and periodate oxidation as discussed in Example 9 below.

Table I. *In vitro* capacity of Il-1ra and PEG_{20kD}-conjugates to inhibit IL-1β induced PG_2 production (ng/mL) in two fibroblast cell lines.

Reagent	Dermal fibroblasts		Synovial fibroblasts	
Culture medium	325 ± 85		239 ± 30	
Buffer without protein	317 ± 69		276 ± 67	
Il-1ra	8.6 ± 3	(100%)	< 4.8	(100%)
Il-1ra(tr)	21.8 ± 6	(96%)	< 4.8	(100%)
PEG_{20kD}-IL-1ra(tr)	219 ± 37	(32%)	98 ± 16	(66%)
PEG_{20kD}-des(MRP)Il-1ra	--		48 ± 14	(84%)

The values are means ±S.D. (n-3). The figures in brackets give a nominal value for the % biological activity of the derivative concerned, even though this initial assay was not carried out on the linear part of the dose-response curve. "--" is not determined "tr" is transaminated form.

As shown in Figures 5A, 5B, and 5C (dose-response curves), the Il-1β effect was inhibited in a dose-responsive manner by all conjugates, and an IC_{50} value (concentration necessary to block 50% of Il-1β response) was approximately determined: the values were approx. 13, 10 and 9 ng/mL for PEG_{5kD}-Il-1ra (Figure 5A), PEG_{10kD}-Il-1ra (Figure 5B), and PEG_{20kD}-Il-1ra (Figure 5C), respectively. Conjugates were prepared by transamination method. The IC_{50} value for authentic Il-1ra was found to be about 6 ng/mL. The necessary ratio of Il-1ra to Il-1β which can be deduced from these results, though higher with the PEG derivatives than with the native protein, remains in all cases in the range 10 to 500, the range described in the literature for Il-1ra (also designated as the pharmaceutical Antril by

Synergen, Inc.) with different cell lines (Arend et al., 1990, J. CLin. Invest. 85:1694-1697).

Pharmacokinetic data. Antril and its derivatives were labeled by the well-known Chloramine-T method (Hunter, W.M. and Greenwood, F.C. (1962) Nature, 194:495-496). The protein (about 0.5 mg) was dialyzed against a 0.1M Tris. HCl, 0.15M NaCl, pH 7.0 buffer, and concentrated to about 300 μl. To this solution were then added 500 μCi NaI and 30 μl of a 1% (w/v) chloramine T solution prepared in the same buffer as that for the protein. Incubation was terminated after 90s by the addition of 30 μl of a 5% (w/v) sodium metabisulfite solution and 25 μl of a 50% (w/v) KI solution. The radioiodinated protein was separated from free ^{125}I on a GF5 column (Pierce) equilibrated in PBS and previously washed with the same buffer containing 0.1% bovine serum albumin. The specific radioactivity thus obtained was in the range of 100-300 μCi/mg protein.

Female Wistar rats obtained from Iffa-Credo (L'Arbresle, France), weighing between 150g and 200g, were used. Approximately 50 μg of ^{125}I-labeled protein were injected as a bolus in the tail vein and samples of blood collected from the tail at selected times (as near as possible to 3, 10, 30 min, 1, 3, 7 and 24h). The samples were weighed to determine their exact volume, and their radioactivity measured. The curves of relative blood levels for Il-1ra, PEG_{10kD}-Il-1ra and PEG_{20kD}-Il-1ra given intravenously to the rats are compared in Figures 6A and 6B. As can be derived from the figures, the apparent first $T_{½}$ of Antril (see the solid lines) was very short, and the apparent second $T_{½}$, though much longer, did not therefore have as strong an influence on the area under the pharmacokinetic curve. Most importantly, even in these preliminary experiments, there are appreciable differences between the apparent first $T_{½}$ values for the two derivatives and the that for Antril. The apparent first $T_{½}$ value for both the derivatives were significantly longer than that for the unmodified protein. A manual fit to these curves gave apparent first $T_{½}$ values of approximately 3 min for authentic Il-1ra, 14 min. for PEG_{10kD}-Il-1ra, and 20 min. for PEG_{20kD}-Il-1ra.

No detectable differences were observed in the apparent second $T_{1/2}$ amongst all three proteins, and in all animals less than 2% of the initially observed radioactivity remained in circulation after 24h.

It is clear from the results presented herein that by increasing the polymer mass, either by increasing its length or, as discussed above, by adding multiple copies of the polymer at one site (via the multi-linker format) one can increase the circulating half-life of a conjugate while not causing a deleterious effect in its activity. This result of the site-specific modification methods and reagents of the present invention sharply contrast results often seen using methods in which the target molecule is randomly labelled with polymer, such as at lysine residues. Unlike with previous technologies, using the reagents and methods of the invention, one would expect a further increase of $T_{1/2}$ without an excessively severe additional decrease in specific biological activity. For example, adding a 40 kD polymer to Antril (either directly or as obtained using smaller but linked polymers) would produce a further increase in the apparent first $T_{1/2}$ without a sudden, crippling loss of specific biological activity. Indeed, using the methods of the invention, the trend of specific biological activity as a function of M_r of the added PEG was observed to be in the other direction (compare Figures 5A, 5B and 5C). Moreover, as presented herein, the use of a multi-linker can achieve similar effects as those observed for a single, linear polymer of molecular weight unexpectedly greater than that of the multi-linker. For example, when two PEG chains were put on a multi-linker and placed site-specifically at the N-terminus of Il-8, the resulting derivative $PEG_{2\times5kD}$-Il-8 had a gel-electrophoretic behavior almost equivalent to that for a derivative with an apparent molecular radius corresponding to the addition of a 20kD PEG chain (see Figure 1).

EXAMPLE 9. Site-Specific Modification and Conjugation of NH_2-Terminal Residue of a Protein: Modification by Enzymatic Cleavage/Periodate Oxidation and Conjugation of IL-1-ra.

Enzymatic cleavage of Met-Arg-Pro and periodate oxidation.

Removal of the three N-terminal residues to reveal the serine can also be considered for the conjugation of the functionalized polymers according to the methodology

already described in Examples 6 and 7. Proline specific endopeptidase (Seikagaku Corp., Tokyo) was used for the cleavage of the N-terminal tripeptide. Digestion was shown to effectively occur on the carboxyl side of the Pro^2 residue, but was very slow.The protein was therefore incubated 20h at 37°C at a 30mg/ml concentration in a 50mM sodium phosphate buffer, pH 7.0 with an enzyme/substrate ratio of 1/10. The protein was then isolated by gel filtration on the BioSep-SecS-2000 Phenomenex column and a aliquot purified by HPLC for the characterization by ESI-MS. Three series of consecutive peaks corresponding to different analogues could be identified: the major one (16874.5±5.4D) corresponding to the expected product *des*(Met-Arg-Pro)Il-1ra (calculated value, 16872.1 D); and two minor ones, one (16785.1±1.5 D) corresponding to the *des*(Met-Arg-Pro-Ser)Il-1ra analogue (calculated value, 16785.0 D), and the other (17125.9±2.1 D) corresponding to *des*-MetIL-1ra (calculated value, 17126.4 D). No trace of the native protein (17257.6 D) could be detected. These results are consistent with the presence of a contaminating aminopeptidase in the commercial enzyme. Nevertheless, this method appears as a good means to reveal a sufficient amount of the serine in position 3 to perform periodate oxidation and polymer conjugation. The protein was oxidized using a 5-fold excess of $NaIO_4$ over the truncated Il-1ra protein solubilized in a 1% NH_4HCO_3 buffer, under the same conditions as described in Example 6, assuming that 50% of the protein material had a N-terminal serine.

Conjugation to the polymer. After extensive dialysis against a 0.1M NaOAc buffer, pH 4.6, the modified protein was mixed with a 10 fold excess of PEG_{20kD}-NH-CO-CH_2-O-NH_2 and the pH brought to 3.6 with acetic acid. After 40h incubation at room temperature, the conjugation product was isolated by hydrophobic interaction chromatography. The coupling reaction was shown to be cleaner than with the transaminated protein.

In vitro activity of conjugates. The in vitro activity of representative conjugate PEG_{20kD}-des(MRP)Il-1ra is presented in Table 1 above.

EXAMPLE 10. Functionalization of Methoxypolyethylene Glycol: Synthesis of Multivalent Linker (PEG-aminoethyl)$_2$-N-(CH_2)$_2$-NH-CO-CH_2-O-NH_2,

This multivalent linker is comparable to that described in Example 3, and was obtained by acylation of Tris-(2-aminoethyl)amine with one equivalent of Boc-NH-O-CH_{22}-CONSu in DMSO. The monosubstituted derivative was isolated by HPLC. characterized by. ESIMS (calcd. M+H, m/z 319.4, found m/z 320.6). The material was solubilized in 10mM HCl and lyophilized, and further acylated with PEG_{5kD}-COOH (1.2 molar excess over remaining amino groups) in DMF, in the presence of equimolar amounts of hydoxybenzotriazole and DCC. The solution was stirred overnight at room temperature and the fully acylated derivative was purified after extensive dialysis against distilled water by ion exchange chromatography on DEAE-A25 and CMC-25 and finally deprotected by treatment to get the following derivative:

$$\begin{array}{l} \qquad\qquad\qquad\qquad (CH_2)2\text{-NH-CO-PEG} \\ \qquad\qquad\qquad\qquad\quad | \\ H_2N\text{-O-}CH_2\text{-CONH-}(CH_2)_2\text{-N} \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad (CH_2)_2\text{-NH-CO-PEG} \end{array}$$

Conjugation to oxidized Il-8 was carried out under the same conditions than used for the linear PEG polymer, and the resulting derivative isolated according to the same procedure, as shown in Figure 1.

EXAMPLE 11. "One-Pot" Modification and Conjugation Reactions.

As demonstrated in the above reactions, side reactions during oxime formation were not a problem. For example, compare lanes 1 and 2 of Figure 3. However, "one-pot" reactions would nevertheless be desirable in order to minimize handling, and thus yields, contamination, equipment, time, etc., as well as provide for more reproducibility and uniformity amongst reactions, decrease labor time and costs, and facilitate development of larger scale and commercial production, as well as simplify use if diagnostic methods or kits. IL-8 was mixed at the same time with both periodate and an aminooxy compound (N^{α}-Aminooxyacetyl, N'LC-biotinyl-lysine), and after an appropriate reaction period, with no work-up whatsoever, the wanted product-- IL-8 specifically biotinylated at its N-terminus--was identified. Surprisingly, the reaction progressed at pHs 8, 6.5 and 4.6. At 6.5 the generation

of product, with approximately quantitative production of the wanted molecule (as identified by mass-spectrometry), was achieved in about 3 hours. The reactions were somewhat slower and less quantitative at the other two pHs. The result was surprising since, for example, periodate oxidation requires a deprotonated amino group, and is therefore favored at alkaline pH, whereas oxime formation is more rapid at acid pH. Additionally, the aminooxy compound, necessarily present in considerable excess, might have been expected to have destroyed the periodate.

In a typical experiment conducted at room temperature (about 20°), 20μl of a solution of Il-8 (2.5 mg in 250μl of 40mM phosphate (Na) pH 6.5) were used. To this solution was added first 2μl of a 41.5 mM of $NaIO_4$, followed immediately, with mixing, by 17μl of a 10mM aqueous solution of N$^{\alpha}$AoA, N$^{\epsilon}$-LC Biotinyl Lysine (prepared as below). After three hours 2μl of the reaction mixture was quenched with 4μl of 10M aqueous ethylene glycol. After further 10 minutes the quenched solution was rapidly diluted in 500 μl 0.1% aqueous trifluoroacetic acid. (This was done to minimize any further oximation reaction, by dilution.) The whole sample was analyzed by reverse phase HPLC. The only substantial peak eluted at the known position of the wanted biotinyl-Il-8 derivative. The product of a large scale reaction when isolated gave the expected mass or electrospray mass spectrometry (calculated m/z 8891.8; found m/z 8892.8 ± 1.2).

The AoA-LC biotinyl Lysine was made from N$^{\alpha}$(BOC-AoA)Lysine and N-hydroxysuccinimido ester of LC (long chain) biotin (which was obtained from the Pierce Chemical Corp). N$^{\alpha}$(BOC-AoA)Lysine was made by a two step process as follows. First, *N-α-tertButyloxycarbonylaminooxyacetyl, N-ε-trifluoroacetyl-Lysine was obtained by* adding to 371 mg of N-ε-trifluoroacetyl-lysine (NovaBiochem, 4448 Laefelfingeu, Switzerland) suspended in 3 mL of DMSO was added, a suspension in 1 mL of DMSO of 576 mg of the N-hydroxysuccinimido ester of aminooxyacetic acid, prepared as described previously (Pochon, et al. (1989) Int. J. Cancer 43:1188-1194), modified as described (Vilaseca et al. (1993) *Bioconjugate Chem. 4*:515-520). N-ethylmorpholine was then added to the suspension, with mixing, until the apparent pH as indicated externally on moist narrow-range pH paper was about 8. The suspension clarified almost immediately upon adjustment to pH 8, and the resulting solution was left

overnight at room temperature. Subsequent thin-layer chromatography *(Vilaseca et al. (1993) Bioconjugate Chem. 4*:515-520) of a small sample showed that little or no ninhydrin-positive material remained. Residual hydroxysuccinimide ester in the reaction mixture was destroyed by dilution with an equal volume of water and incubation at 37°C for 1 hour. The mixture was then further diluted with 32 mL water, cooled to 0°C, and brought to an apparent pH of 3.0 (glass electrode) with acetic acid. The solution was then divided in two, and each half placed on a Chromabond 1000 mg (Machery-Nagel, Düren, 52348, Germany) equilibrated with 0.1% aqueous TFA. Each Chromabond was then washed with 20 mL of the same solution, and eluted with 4 mL of a mixture of 0.1% TFA:acetonitrile, 4:6 (v/v). Acetonitrile was removed from the eluates in a current of filtered air, and the remaining liquid removed by vacuum centrifugation. Second, the trifluoroacetyl group was removed from the ϵ-amine of the lysine by adding 3 mL of water to each of the dried down eluates, cooling to 0°C, then adding 330 μL of piperidine. The mixture was maintained in an ice bath with occasional agitation for 3 h. The reaction was stopped by the careful addition of 500 μL of glacial acetic acid, and solutions could be stored frozen at this point. To continue the procedure, each mixture was diluted to 10 mL with water and the pH adjusted with acetic acid to pH 3.0 (glass electrode) if necessary. The mixtures were applied to two Chromabonds as before, except that (a) elution of the wanted fraction was carried out with 4 mL of a mixture of 0.1% TFA:acetonitrile, 7:3 (v/v) and (b) for each Chromabond the material passing through unchecked was pooled with the subsequent 20 mL wash fraction and passed a second time over the same Chromabond after re-equilibration. For each Chromabond, the two eluates were pooled and dried down to give approx. 40 mg each of the desired product N-α-tertButyloxycarbonylaminooxyacetyl-Lysine.

The AoA-LC biotinyl Lysine was made by first dissolving 15.3 mg of the N$^{\alpha}$(BOC-AoA)Lysine in 478 μl of DMSO and adding 5μl N-ethylmorpholine. To this were added 53.2 mg of the biotin compound N-hydroxysuccinimido ester of LC (long chain) biotin dissolved in 478 μl DMSO. If either component was reluctant to dissolve before mixing, all went into solution afterwards. After 18 hours at room temperature any excess active ester was destroyed by adding 956 μl

NaHCO3 (1% in water) and waiting for 2 1/2 hours. The reaction mixture was diluted with 10μl H_2O acidified by the careful addition at 0°C of 60μl acetic acid (to pH 3). The mixture was loaded on a Chromabond separation cartridge equilibrated in 0.1% TFA, the cartridge washed with 20 ml 0.1% TFA, then 4 ml of CH_3CN/0.1% TFA (1:4 v/v). The wanted product was diluted with 4ml of CH3CN/0.1% μl TFA (2:3 v/v) and dried down. Yield was 23.8 mg.

The dried fraction was dissolved in 200 μl CH_3CN plus 1800 μl 0.1% TFA. The solution was clarified if necessary by centrifugation and 1 ml was injected at a time on the C8 analytical column described above. The column had been equilibrated with 20% of the second solvent described above, and this was brought to 40% in the first five minutes after injection. The value was then taken to 80% over the next 100 minutes. The wanted product was the principal peak on this latter part of the gradient. The peak was dried down. Mass spectrometry showed the expected mass. The BOC group was removed by dissolving 1 mg in 200μl TFA. After 45 minutes at 20° the solution was dried down and used without a further purification.

EXAMPLE 12. Functionalization of Methoxypolyethylene Glycol: Synthesis of PEG-CHO.

Though several methods have already been described for oxidizing the terminal hydroxyl group (Wirth et al., (1991) Bioorganic Chem. 19, 133-142; Harris M.M. et al. (1984) J. Polym. Sci. Polym. Chem. Ed. 22, 341), the aldehydic function was introduced by acylating PEG-NH_2 with carboxy (benzaldehyde)OSu. PEG-NH_2 (1 gram, 50 μmol) and carboxy (benzaldehyde)OSu (62 mg, 250 μmol) were dissolved in DMSO (4 ml), the apparent pH adjusted to 8-9 with M-Methylmorpholine and the mixture stirred overnight. Acylation was controlled by the standard ninhydrin analysis. The solution was then diluted with 5 volumes of distilled water, extensively dialyzed against water and finally lyophilized.

EXAMPLE 13. Site-Specific Modification and Conjugation of Cysteine Residue of a Protein: Modification and Conjugation of rh-G-CSF at Cysteine17.

Another approach to modify G-CSF site-specifically was alkylation of Cys^{17} with a linker bearing the AoA function in order to place a reactive AoA at

this position. In the case of G-CSF there was only a single Cys residue. For this purpose was used the linker compound

$$Boc\text{-}NH\text{-}O\text{-}CH_2\text{-}COHN\text{-}(CH_2)_2\text{-}NH\text{-}CO\text{-}CH_2\text{-}Br$$

The Boc group was removed and the linker rapidly added at a 20mM concentration to rh-G-CSF solubilized in a 0.1M phosphate buffer (Na), pH 7.0, 5mN EDTA and 6M GuHCl. After 30 min. incubation, the solution was acidified. The alkylated G-CSF was purified by reverse phase HPLC and characterized by mass spectrometry (calcd. m/z 18971.8, found, m/z 18978.2+7.8). The protein was then resolubilized in acetate buffer (0.1M Na), pH 4.6 containing 6M GnHCl and reacted with a PEG-CHO (from Example 12 above). The extent of polymer conjugation was comparable to that obtained in the two-step coupling at the N-terminus described in the previous example. If the alkylation with the preceding linker is performed at higher pH values (e.g. pH 8.0, pH 9.0) histidine and methionine residues will also be modified.

EXAMPLE 14. Site-Specific Modification and Conjugation of a Protein: Modification and Conjugation of rh-G-CSF.

A small bivalent tag, containing a group reactive with a functionalized polymer of the invention, can be added to the protein in a large excess and will therefore react rapidly with the created glyoxylyl function in the protein. In a second step the functionalized polymer is added to achieve conjugation to the protein via the tag. This two-step approach is useful, for example, when one wishes to avoid or minimizes both steric problems and those that might be caused by side reactions. A illustrative small, bivalent tag is a bisaminooxy tag. $H_2N\text{-}O\text{-}CH_2CO\text{-}Lys(CO\text{-}CH_2\text{-}O\text{-}NH_2)$ was synthesized by the attachment of Boc-AoA-ONSu to Lys-OMe, followed by NaOH treatment and TFA deprotection (calcd. M+H, 293:1; found M+H 293.9). To complement an AoA group on the protein, a functionalized polymer having a reactive carbonyl group was used for conjugation. $PEG\text{-}NH_2$ was reacted with carboxy-(benzaldehyde)-ONSu to give a polymer with the required aldehydic function for reaction with the AoA-tagged G-CSF, yielding the final product:

$$PEG\text{-}NH\text{-}CO\text{-}C_6H_4\text{-}CH{=}N\text{-}O\text{-}CH_2\text{-}CO\text{-}LysCO\text{-}CH_2\text{-}O\text{-}N{=}CH\text{-}CO\text{-}\textit{des}Met^1mG\text{-}CSF$$

EXAMPLE 15. Synthesis of a multiaminooxy linker and conjugation to the protein.

Tris(2-aminoethyl)amine, used in Example 10, was acylated with 3 equivalent of Boc-NH-O-CH_2-COOSu in DMSO. The fully substituted derivative was isolated by HPLC, characterized by ESIMS (calcld. M & M, m/z 665.5, found m/z 665.3). The material was deprotected by 1h TFA treatment and TFA removed by rotary evaporation. The material was resolubilized in water and lyophilized then reacted in a 15 molar excess (to avoid crosslinking) with oxidized II-8 in 0.1M AcoNa, pH 3.6. The conjugation product (bearing two free AoA groups) was isolated by HPLC and characterized by ESIMS (calcld. M & H, m/z 8698.6, found m/z 8696.0 $\pm$ 0.5).

This conjugate can be reacted with PEG-CHO to yield a two-polymer armed derivative, i.e. a bi-polymer-containing functionalized polymer.

Other multiarm, i.e. multi-polymer-containing functionalized polymer, structures can be constructed, especially if the aim of the polymer conjugation is to decrease immunogenicity of the protein. Indeed, a linker bearing 3 to 10 arms, preferably 4 to 6, would be more appropriate. Such construction can be obtained by linking, in a first reaction, to the protein a peptidic linker of this structure:

$$H_2N\text{-}O\text{-}CH_2\text{-}CO\text{-}X\text{-}(Lys(Ser))n$$

or the corresponding dendrimeric, arborescent form with N-terminal serine residues, where X is a peptidic or nonpeptidic structure which facilitates separation of the conjugation product from unreacted protein and n is the number of attached polymers as defined above. Additional spacer groups can be introduced between lysine residues.

In a second reaction, the serine residues of the introduced linker are oxidized, and then reacted as described in preceding examples, with PEG-AoA functionalized polymers, to covalently attach n number of chains to the protein.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A functionalized polymer, comprising an organic polymer covalently attached to an amino-oxy oxime-forming group.

2. The functionalized polymer of Claim 1, wherein the organic polymer is attached to two amino-oxy groups.

3. The functionalized polymer of Claim 2, wherein the organic polymer, having a first terminus and a second terminus, is covalently attached to a first amino-oxy group at its first polymer terminus and to a second amino-oxy group at its second polymer terminus.

4. The functionalized polymer of Claim 1, wherein the polymer is a water-soluble polymer.

5. The functionalized polymer of Claim 4, wherein the water soluble polymer is selected from the group consisting of dextran, dextran sulfate, P-amino cross-linked dextrin, carboxymethyl dextrin, cellulose, methylcellulose, carboxymethyl cellulose, starch, dextrines, hydroylactes of starch, polyalklyene glycol, heparin, fragments of heparin, polyvinyl alcohol, polyvinyl ethyl ethers, polyvinylpyrrolidone, α,β-Poly (2-hydroxyethyl)-DL-aspartamide, polyoxyethylated polyols, and polynucleotides.

6. The functionalized polymer of Claim 5, wherein the polyalklyene glycol is selected from the group consisting of straight, branched, disubstituted, or unsubstituted polyalklyene glycol, polyethylene glycol homopolymers, polypropylene glycol homopolymers, and copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group.

7. The functionalized polymer of Claim 7, wherein the polymer is polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG).

8. The functionalized polymer of Claim 5, wherein the polyoxyethylated polyol is selected from the group consisting of polyoxyethylated sorbitol, polyoxyethylated glucose, and polyoxyethylated glycerol.

9. The functionalized polymer of Claim 1, wherein the polymer has a molecular weight average of about 200 to 200,000.

10. The functionalized polymer of Claim 9, wherein the polymer has a molecular weight average of about 400 to 50,000.

11. The functionalized polymer of Claim 10, wherein the polymer has a molecular weight average of about 2000 to 40,000.

12. The functionalized polymer of Claim 5, wherein the molecular weight average of dextran or its derivatives is 10,000 to 500,000.

13. The functionalized polymer of Claim 5, wherein the polymer has the formula
R1-O(R2-O)nR2-R3
where n is an integer between 5 and 2,000, R2 is a lower alkyl group which is straight, branched, disubstituted, or unsubstituted, and (a) one of R1 and R3 comprises an amino-oxy oxime-forming group and the other of R1 and R3 is hydrogen, -CH3, or a protective group, or (b) both R1 and R3 comprise an amino-oxy oxime-forming group, and where either or both of R1 and R3 optionally comprise a spacer group.

14. The functionalized polymer of Claim 13, wherein the protective group is an alkyl group.

15. The functionalized polymer of Claim 14, wherein the protective group has between 1 and 10 carbons.

16. The functionalized polymer of Claim 15, wherein the protective group is methyl.

17. The functionalized polymer of Claim 13, wherein n is a integer between 10 and 1000.

18. The functionalized polymer of Claim 13, wherein the amino-oxy oxime-forming group comprises -X-O-NH_2, wherein X is a spacer group comprising -NH-CHO-R4- and wherein R4 is attached to -O-NH_2 and is a substituted or unsubstituted linear, branched, or cyclic lower alkyl.

19. The functionalized polymer of Claim 18, wherein R4 is -CH2-.

20. The functionalized polymer of Claim 1, having the formula $(P)_mL$-X-O-NH_2 or $(P)_mL$-X-C(R)O, wherein P is the organic polymer, m is an integer from 2 to 10, X is a spacer group, R is hydrogen or substituted or unsubstituted C1-C10 linear or branched alkyl group, and L is a multi-valent linking group to which each P is covalently linked, and wherein the valency of L is at least m+1.

21. The functionalized polymer of Claim 20, wherein X comprises -CH_2- or -CHOH- in the formula (P)mL-X-O-NH_2.

22. The functionalized polymer of Claim 21, wherein X comprises -CO-CH_2- or -NH-CO-CH_2- in the formula (P)mL-X-O-NH_2.

23. The functionalized polymer of Claim 20, wherein X comprises -CH_2-, -CO-, or -CHOH- in the formula (P)mL-X-C(R)O.

24. The functionalized polymer of Claim 20, wherein L comprises a tri-amine of the formula N(R5-NH-)$_3$ wherein R5 comprises a substituted or unsubstituted aliphatic or aromatic group.

25. The functionalized polymer of Claim 24, wherein R5 is selected from the group consisting of phenyl or C_1-C_{10} alkylene moieties, C_1-C_{10} alkyl groups, or a combination thereof, an amino acid peptide, an oligonucleotide, an oligosaccharide, a lipid chain or a combination thereof, and may contain heteroatoms.

26. The functionalized polymer of Claim 24, wherein R5 is -CH2-CH2-.

27. A method of systematically modifying the Stokes radius of an organic target macromolecule, comprising the steps of

(a) obtaining a site-specifically-functionalized target macromolecule comprising a first oxime-forming group,

(b) obtaining a series of functionalized organic polymers differing from each other in the series in topology but not molecular weight(average) comprising a second oxime-forming group complementary reactive to the first oxime-forming group, and then

(c) conjugating the functionalized target macromolecule separately with each functionalized polymer via oximation to obtain a series of conjugated polymers, wherein steps (a) and (b) are performed in any order.

28. The method of Claim 27, which further comprises the step of

(d) identifying a change in a biological or physical property of a conjugated polymer of step (c).

29. The method of Claim 27, wherein the second oxime-forming group comprise -O-NH_2.

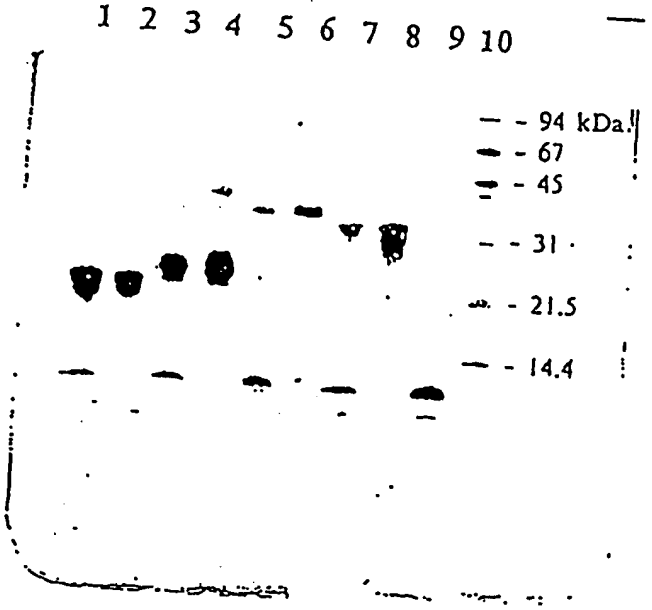


FIGURE 1

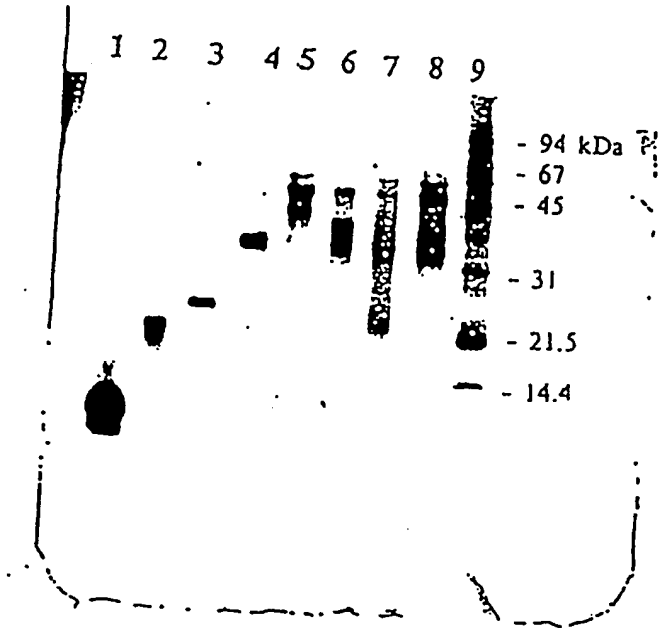


FIGURE 2

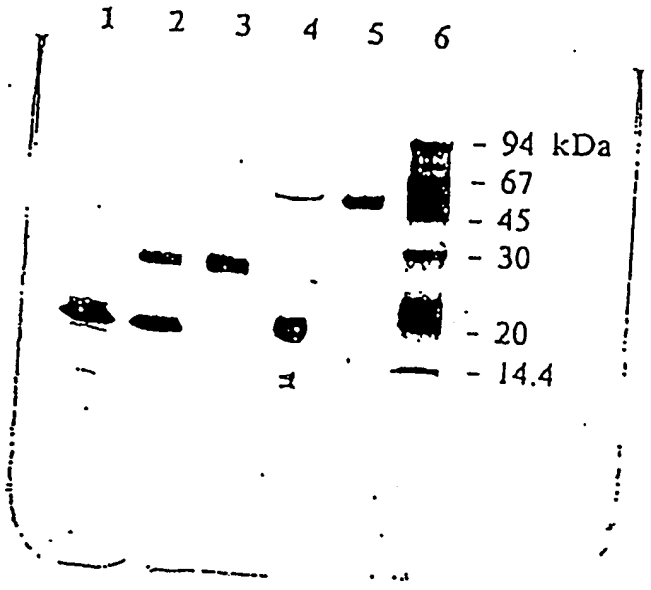


FIGURE 3

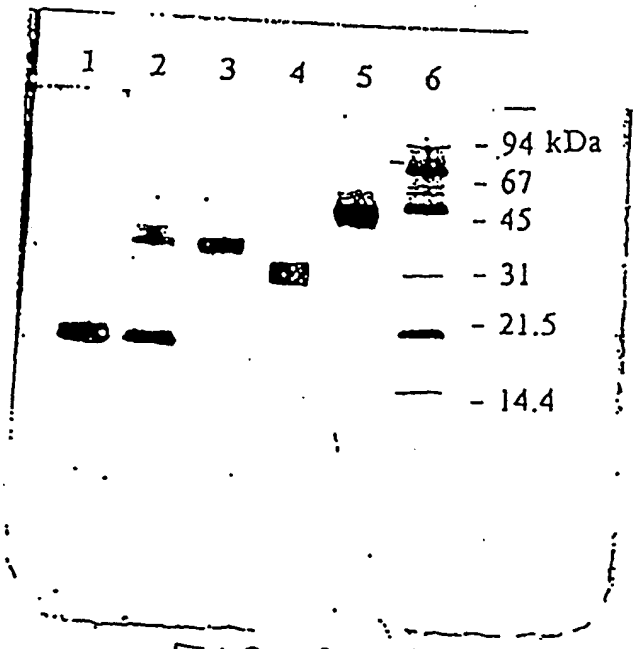


FIGURE 4

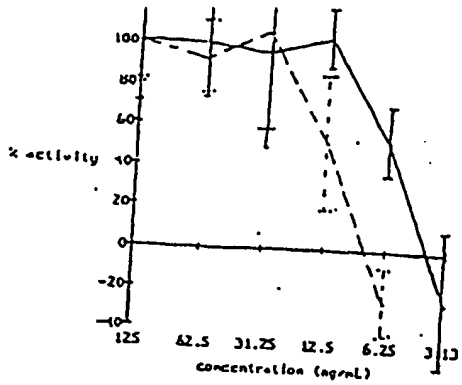


FIGURE 5A

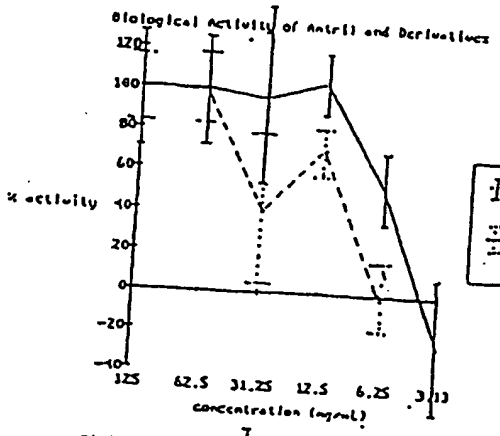


FIGURE 5B

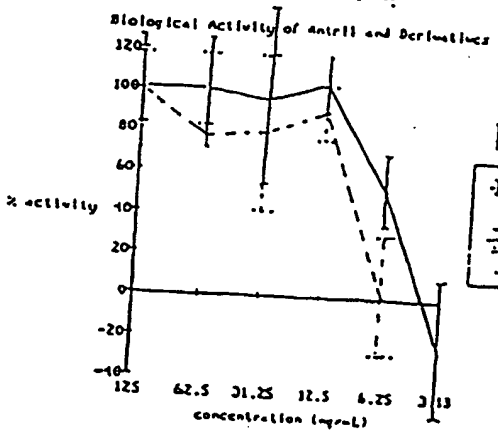


FIGURE 5C

FIGURE 6A

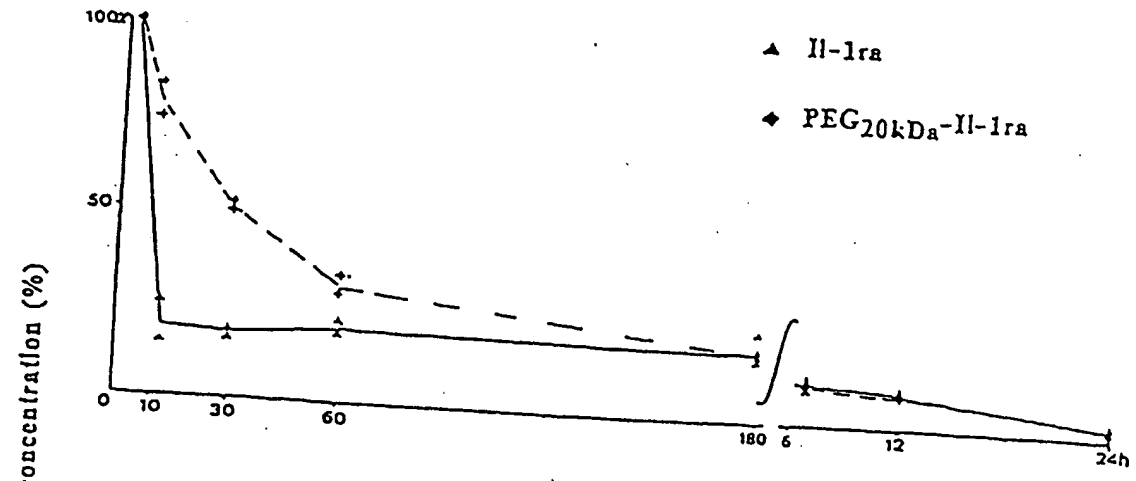


FIGURE 6B

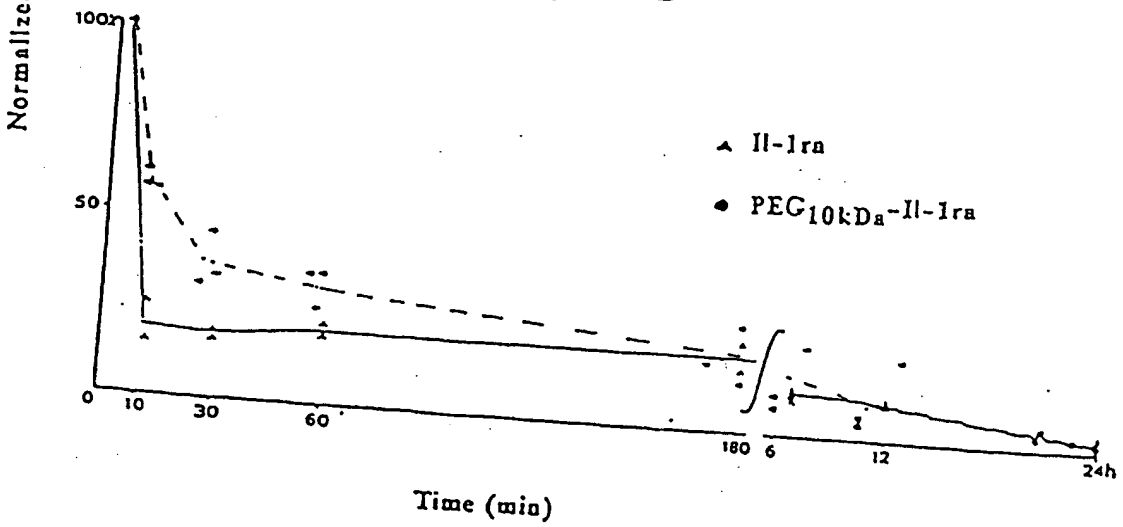
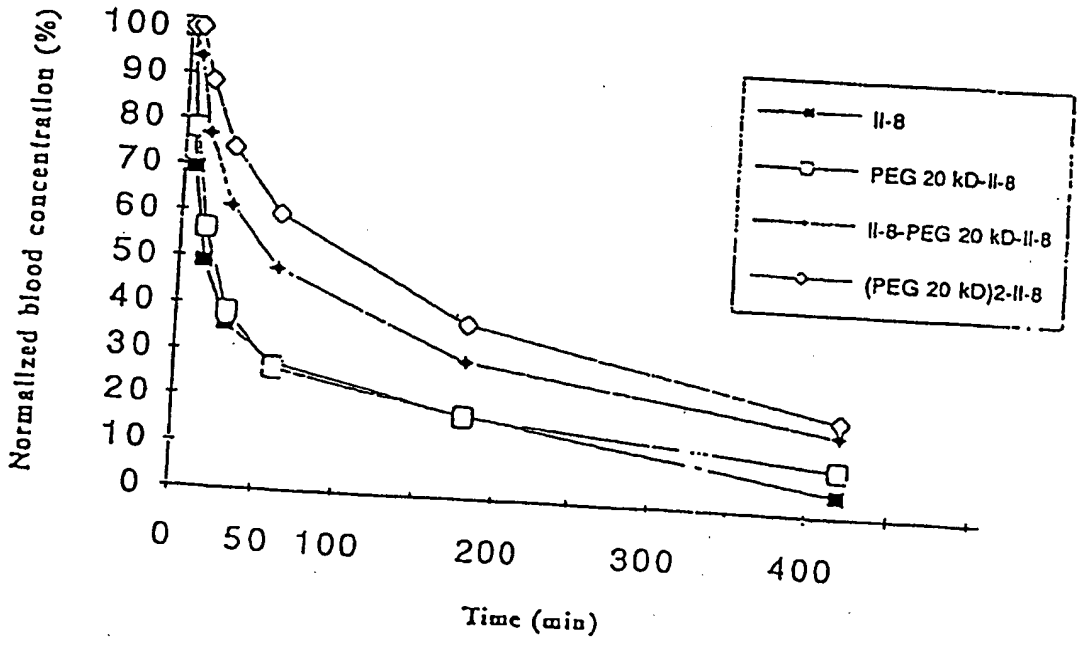


FIGURE 7


Normalized blood concentration (%)
100
90
80
70
60
50
40
30
20
10
0
0
50
100
200
300
400
Time (min)
Il-8
PEG 20 kD-Il-8
Il-8-PEG 20 kD-Il-8
(PEG 20 kD)2-Il-8